Functional analysis of phototropin in *Chlamydomonas reinhardtii*

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Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbständig ohne fremde Hilfe verfasst und nur die angegebene Literatur verwendet zu haben.

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Yinghong Lu 卢颖洪

2. Dezember 2005

Zusammenfassung

In höheren Pflanzen vermittelt der Blaulicht-sensitive Photorezeptor Phototropin verschiedene Reaktionen wie Phototropismus, Chloroplastendrehung und die Öffnung von Schließzellen. Alle Reaktionen haben mit der Optimierung der pflanzlichen Lichtaufnahme und damit einer angepaßten Photosynthese sowie der Vermeidung von Lichtschädigung zu tun. In C.*reinhardtii* haben alle Phototropinreaktionen mit dem Sexualleben dieser Alge zu tun, d.h. Gametogenese, sexueller Kompetenz sowie der Keimung von Zygoten.

Diese Doktorarbeit wurde Ende 2001 begonnen und verlief parallel zu den Arbeiten von Huang. Im Unterschied zu den Ergebnissen von Huang war das *Chlamydomonas* Phototropin in meinen Händen unlöslich. Das Protein war nicht komplett membranassoziiert, sondern ein Teil des Proteins blieb immer löslich. Die Phototropinmenge sowie die Verteilung waren in vegetativen Zellen, die unter Stark- oder Schwachlicht gewachsen waren, unterschiedlich.

Deshalb wurde für diesen Unterschied Licht als wesentlicher Faktor verantwortlich gemacht. Jedoch zeigen verschiedene Stämme bei vegetativem Wachstum unter identischen Lichtbedingungen unterschiedliche Phototropinmengen. Das deutet auf weitere Faktoren hin, die Konzentration und Verteilung von Phototropin beeinflussen.

In *Chlamydomonas* wurde neben dem Volllängenprodukt noch eine c-terminal verkürzte Phototropinvariante gefunden. Licht wurde als Verursacher der Verkürzung identifiziert. Aber nur lange Belichtungen von ca. 48 h führten je nach Intensität zu klaren Abbaumustern. Für das Studium der Beteiligung des Phototropins am Sexualleben von *Chlamydomonas*, sollte ein Phot- Stamm generiert werden. Dazu wurde ein RNAi-Konstrukt hergestellt, mit dem es möglich war, im Stamm cw15 arg- A das Phototropin bis auf 10% des Originalniveaus zu reduzieren. Leider funktionierte das Konstrukt in anderen Stämmen nicht zuverlässig. Im Stamm CC32pab1mt(+) konnte nur ein Klon mit einer Reduktion auf 15% des Originalniveaus erreicht werden. Außerdem war die Silencing-Effizienz stark von den Wachstumsbedingungen abhängig. Die beste Reduktion wurde bei niedrigen Lichtintensitäten gefunden.

Es wurde ein weiterer Kreuzungstest etabliert, der für die Analyse der Zygotenkeimung verschiedene Vorteile gegenüber bekannten Tests liefert. Aus den durchgeführten Tests konnte geschlossen werden, dass Licht auch ein wesentlicher Faktor für die Zygotenkeimung ist. Bei mittleren Lichtintensitäten keimen Zygoten mit wenig Phototropin später. Starklicht kann diesen Mangel weitgehend kompensieren.

Zur biochemischen Analyse des Phototropins in vitro war die Expression eines markierten Phototropins notwendig. Zur Analyse des Phototropinabbaus und für die spätere Reinigung wurde auch versucht, Phototropin in verschiedenen Gastorganismen zu exprimieren. In dieser Arbeit wurde Phototropin in Xenopus Oocyten und Diatomäen exprimiert. Diese Versuche haben bestätigt, dass das Phototropinabbauprodukt vom selben Gen wie das Volllängenprotein resultiert. Durch Expression der Phot-Mutante S57S/C250S konnte auch gezeigt werden, dass die Aktivierung des Phototropins keine Voraussetzung für den Abbau ist. Erstmalig konnte auch Phototropin als Fusionsprotein in *Chlamydomonas* exprimiert werden. Das Fusionsprotein wurde gereinigt und die Identität massenspektrometrisch verifiziert. Es wurde ein Stamm gefunden, der nur eine verkürzte Variante des Phototropins exprimiert. Das Produkt war besser löslich als die Volllängenversion. Eine Großproduktion sollte für die Reinigung und nachfolgende Kristallisation angesetzt werden. Tandem Affinitätsreinigungen sollten für die Identifizierung von Reaktionspartnern durchgeführt werden.

Acknowledgments

I want to thank my supervisor, Prof. Peter Hegemann. It is because of you that I got to know this tiny but very interesting little creature ---- *Chlamydomonas*. It is under your supervision, I understand more about science research. With your help, I improved myself in many fields. Thank you so much!

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Contents

Abbreviations

Abbreviations	Full name
aa	Amino Acid
Amp	Ampicillin
AR protmoter	*HSP70A* and *RbcS2* promoter
bp	Base Pair
BCA	Bicinchoninic acid
BCIP	5-Bromo-4-Chlor-3-Indolyl-Phosphate
BLE	Zeocin Binding Protein
BSA	Bovine Serum Albumin
cDNA	Complimentary DNA
CIP	alkaline phosphatase
COP	Chlamyopsin
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
EtBr	Ethidium Bromide
FMN	Flavin mononucleotide
g	Gram
GFP	Green Fluorescence Protein
HSA	High salt acetate medium
IFT	Intraflagella transport
IOD	Integrated optical density
M	Molar
min	Minute
mi-RNA	micro interfering RNA
ml	Milliliter
mM	Millimolar
mt	Mating type
NBT	4-Nitroblue-Tetrazoliumchloride

Abbreviations	Full name
NC	Nitro Cellulose membrane
NMM	Nitrogen Minimal Medium
NP40	Nonionicdetergent P-40
nt	Nucleotide
OD	Optical Density
Pab	p-aminobenzoic acid
PAGE	Polyacrylamid Gel Electrophoresis
PCR	Polymerase Chain Reaction
Phot	Phototropin
RdRp	RNA dependent RNA ploymerase
RISC	RNA-inducing silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
SDS	Sodium Dodecylsulphate
Sh Ble	*Streptoalloteichus hindustanus* Bleomycin resistance protein
si-RNA	short interfering RNA
rpm	revolutions per minute
TAP medium	Tris-Acetate-Phosphate medium
TAP method	Tandem Affinity Purification method
TEMED	N,N,N',N' Tetramethylethylenediamine
TEV protease	Tobacco Etch Virus protease
Tris	Tris (hydroxymethy) amino methane
UTR	untranslated region
UV	ultraviolet
V	Volt

1. Summary

In higher plants, phototropin is in charge of phototropism (Liscum, 2002), chloroplast relocation (Wada et al., 2003) and stomatal opening (Schroeder et al., 2001). All its functions are connected with plants' modulation to the surrounding light conditions so that plants can make the best use of light for photosynthesis and dodge harmful strong light. In *C.reinhardtii*, all its reported functions are connected to the sexual life of this green alga, i.e. gametogenesis, maintenance of gamete competence and zygote germination.

This PhD work started at the end of 2001 and went on in parallel with Huang's work. Different from Huang's observation that phototropin was insoluble (Huang et al., 2002), it was found that phototropin existed not only as a membrane associated protein, a portion of phototropin always remained soluble. Phototropin levels and distributions were different between vegetative cells grown in strong light or in darkness. Light was thought to be the essential factor that caused this difference. But, different strains grown vegetatively under same light conditions showed that the levels of phototropin and its distributions varied. This suggested the existence of other factors in the determination of its level and distribution.

A C-terminus degradation product of phototropin was found as a stable component in *C. reinhardtii*. Light was found as a reason that caused the degradation. However, short time of illumination with strong light (up to 2 h) did not evoke the degradation machinery. In light gradient experiment, long time illumination (~48h) with different light intensity showed a clear degradation pattern.

To study phototropin involvement in *Chlamydomonas* sexual life, a Phot1- strain was needed. An RNAi *phototropin* construct was made. It managed to reduce the level of phototropin in strain *cw15 arg- A* down to 10% of its original level. However, the construct did not work properly in other strains. Only one transformant of strain *CC32pab1mt(+)* with a reduction of the phototropin level to around 15% of the original level was found. Under different growth conditions, the silencing efficiency varied. The best silencing result appeared when cells were grown under low light conditions.

A new mating assay was established in this work, which has many benefits over the traditional way of studying zygote germination. The conclusion was drawn from this assay that light was the primary factor that determines zygote germination; under moderate light conditions, zygotes with higher phototropin level would germinate earlier; strong illumination could compensate the difference caused by the low phototropin level in zygote germination.

For phototropin function analysis *in vitro*, a *Chlamydomonas* strain that over-expressed phototropin was in need. To prove that the suspected degradation product originated from the *phototropin* gene and to purify phototropin for crystallization, trials to express *C.r. phototropin* cDNA in different organisms were also made.

Phototropin was expressed in *Xenopus* oocytes and diatom in this work. The expression pattern confirmed that both the full-length and the suspected degradation product did originate from the same *phototropin* gene. It was also found that the degradation was independent on phototropin activation state by expressing *C.r.* Phot1 (C57S, C250S) in oocytes. For the first time, recombinant phototropin got expressed in *Chlamydomonas* by fusion expression strategy. The fusion product was purified and the identity was confirmed by Mass Spectrometry analysis. One strain which expressed only a C-terminus truncation version of the fusion product was found. Compared with the full length product, this mutant had better solubility and was easier to be purified. Large scale of purification should be performed to obtain enough material for crystallographic studies. Tandem affinity purification (TAP) tag should also be performed in *Chlamydomonas* proteomic studies about phototropin.

2. Introduction

2.1 Properties of phototropin

Sunlight, the ultimate energy source of the earth, is of great importance for growth and development of all living creatures. With the alternation of days and nights and the changing of the four seasons, light intensity in every corner of the earth varies and changes permanently. Thus, the ability for living creatures to detect the ambient light change becomes necessary. Not like animals, which use opsin-based visual system to receive information about the change of light, plants managed to develop a set of different nonopsin photoreceptors, which are able to perceive a wide range of light qualities and intensities.

In the range of 400-850nm of the light spectrum, plants have three major classes of photoreceptors, the cryptochromes (cry), the phototropins (phot), and the phytochromes (phy). Among those photoreceptors, cryptochromes and phototropins are in charge of monitoring the blue/ultraviolet (B/UV-A) region of the spectrum, while the phytochromes monitor mainly the red (R) and far red (FR) wavelength.

Figure 2.1.1 Domain features of plant phototropin

The phototropins could be divided into two major regions: the LOV domain region and a Ser/Thr kinase region. Two LOV domains are sensor domains which are in charge of receiving blue light signals and the Ser/Thr kinase domain is the signal output domain. All the phototropins discovered up to now contain two LOV domains and one kinase domain, each LOV domain binds one FMN as a chromophore.

As early as in the late 19 century, Charles Darwin carried the first delicate experiment to study the phototropic behavior of grass coleoptiles (Darwin, 1880). In the book *Power of Movement in Plants*, he first described the bending of grass seedling coleoptiles toward a light source. For about hundred years, this phototropic response still remains a mystery in the biochemical level. The hypothesis, that blue light-dependent protein phosphorylation was an important component in the signal perception and transduction pathway of phototropism, got strong support from the results obtained from Briggs group (Reymond et al., 1992).

Later, a phototropic mutant of *Arabidopsis thaliana*, strain JK224 was found (Khurana and Poff, 1989), which showed only little blue light-induced phosphorylation. Complete lack of blue light-dependent phosphorylation was observed in null mutant of NPH1 (Non-Phototropic Hypocotyl 1) locus of Arabidopsis (Liscum and Briggs, 1995). Subsequent map-based cloning of *nph1* demonstrated that it encodes a 120-kD plasmamembrane-associated Ser/Thr protein kinase (Huala et al., 1997). This protein was first named as

NPH1 since it is the product of *nph1* gene and later changed to Phototropin because its involvement in phototropism.

Phototropin is composed of two LOV domains in the N-terminal part and one Ser/Thr kinase domain in the C-terminal part (Figure 2.1.1). The LOV domains belong to the PAS (Per-Arnt-Sim) domain superfamily. Proteins which contain LOV domains are sensitive to the change of light, oxygen and voltage (Zulin et al., 1997; Taylor and Zhulin, 1999) and are named LOV for that reason. Each LOV domain binds a flavin mononucleotide (FMN) as chromophore. One oxidized FMN is non-covalently bound to one LOV domain, the complex is called LOV^{D}_{477} (Crosson and Moffat, 2001; Swartz et al., 2001).

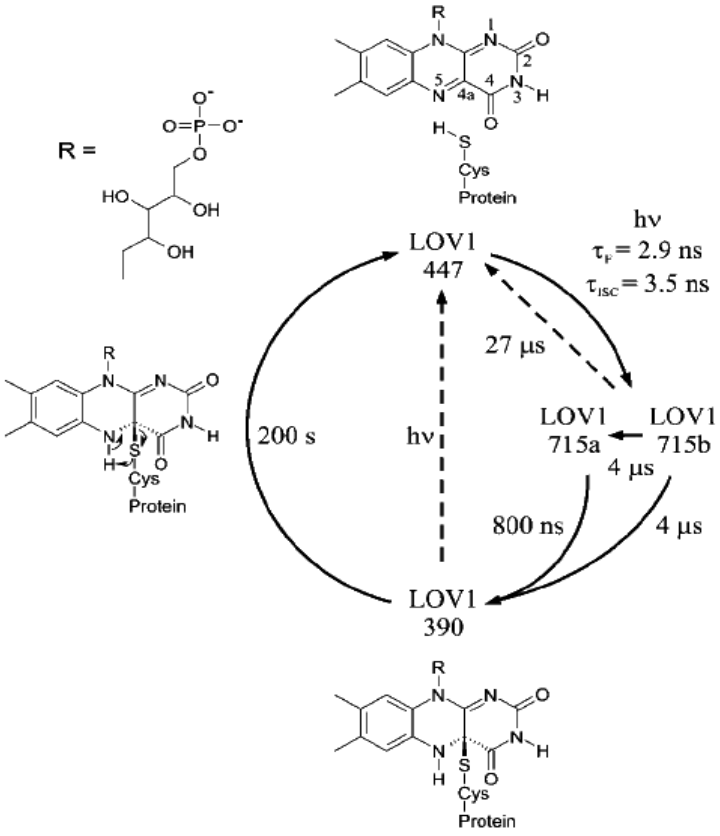


Figure 2.1.2 Photocycle of LOV1 domain from *C. reinhardtii* phototropin (Kottke et al 2003)

When activated by blue light, a photon is absorbed and an excited singlet state is generated, then the singlet state converts to a triplet state $LOV1_{715}$ by intersystem crossing (Swartz et al., 2001; Kennis et al., 2003; Kottke et al., 2003). There are two subspecies of $LOV1_{715}$, namely $LOV1_{715a}$ and $LOV1_{715b}$, both resembling triplet states (Kottke et al., 2003). The N5 atom of the isoalloxazine ring of the excited triplet state accept a hydrogen atom from the cysteine which is present in the highly conserved sequence NCRFLQ (Asn-Cys-Arg-Phe-Leu-Gln) in all LOV domains (Kennis et al., 2003; Corchnoy et al., 2003; Salomon et al., 2000). The protonation of FMN not only stablizes the triplet state but also increases the electrophilicity of the C(4a) atom which triggers the attack from the thiol anion. Thus, a cysteinyl-FMN adduct is formed with a absorption maximum of 390nm (LOV^{S}_{390}) (Swartz et al., 2001; Kennis et al., 2003; Kottke et al., 2003; Salomon et al., 2000). The LOV^{S}_{390} cysteinyl-FMN adduct is thought to be the active signaling form of phototropin (Crosson and Moffat, 2002; Salomon et al 2001). Replacement of cysteine in NCRFLQ with either serine or alanine

banishes the ability of light-dependent formation of LOV^{S}_{390} (Swartz et al., 2001; Kottke et al., 2003).

Arabidopsis phot1 with two cysteine to alanine mutations in NCRFLQ of both LOV1 and LOV2 lost the ability to complement the phot1 null mutant. The two LOV domains seem to play different roles. *Arabidopsis* phot1 with cysteine to alanine mutation in only LOV1 manages to complement the *phot1 null* mutant while *Arabidopsis* phot1 with cysteine to alanine mutation in LOV2 fails to complement the phot1 *null mutant*, which suggest the LOV2 domain is the more important sensor domain in phototropin (Christie et al., 2002). Similar studies about the phot2-dependent chloroplast avoidance response in fern also lead to the same conclusion (Kagawa et al., 2004).

Phototropins, although the name comes from its relationship with phototropism, are not only the sensors for phototropism. It has been shown in *Arabidopsis*, that they are also involved in chloroplast relocation and stomatal opening (Briggs and Christie et al., 2002). In *Arabidopsis*, there are two phototropins (phot1 and phot2) and both of them are involved in the functions mentioned above. As reported originally by Liscum and Briggs (1995), the phot1- mutant lost both hypocotyl and root phototropism in response to low-intensity of blue light. Phot1 is in charge of phototropism in high-intensity and low-intensity of blue light and phot2 mediates phototropism only in high-intensity of blue light (Sakai et al., 2001; Jarillo et al., 2001). In order to make best use of sun light, *Arabidopsis* chloroplasts have an accumulation response to maximize light capturing under low light condition so that it can get enough energy for photosynthesis. Arabidopsis choloroplasts also show an avoidance response to minimize light illumination under very strong light condition so that the photo damage is reduced. Both phot1 and phot2 are in charge of the accumulation response of chloroplasts to low intensity blue light. Phot2 is not as sensitive as phot1 in low light condition. Phot2 level is up regulated by high light condition and is in charge of the avoidance response under high light condition while phot1 still mediates the accumulation response even under the high light condition (Sakai et al., 2001; Jarillo et al., 2001; Kagawa et al., 2001).

2.2 Phototropin in *Chlamydomonas reinhardtii*

In *Chlamydomonas*, a phototropin was discovered in 2001 (Huang et al., 2002). It is a single copy gene and the amino acid sequence has high homology with phototropins from higher plants. The calculated molecular weight is 81.4kD, which is much smaller compared those phototropins from higher plants (~120kD).

Due to the failure of over expressing *C. reinhardtii* phot1-GFP fusion protein, Huang analyzed of the localization of phot-GFP fusion proteins in transiently transformed tobacco BY-2 protoplasts, and found that the fusion product associated with endogenous membranes (Huang et al., 2002). In parallel with the present work, Huang also found that phototropin existed in both cell body and flagella. In cell body, phototropin is

located in the plasma membrane and the microsomal membrane. In flagella, phototropin attaches to the axoneme and is transported in flagella by IFT (intraflagella transport) (Huang et al., 2004).

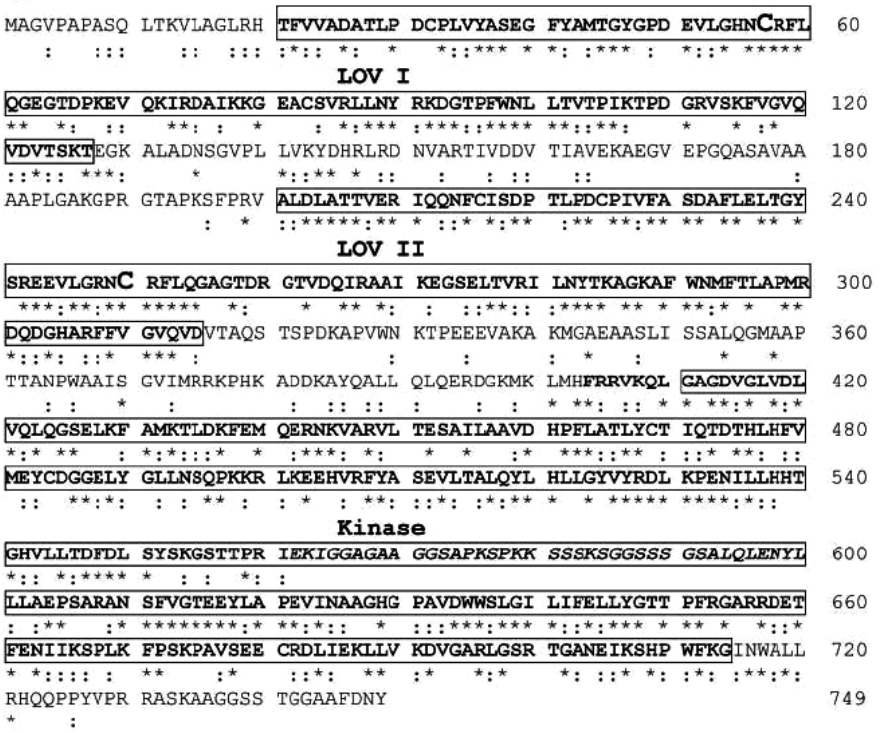


Figure 2.2.1 Amino acid sequence of *Chlamydomonas phototropin* gene (Huang et al., 2002)

The caged characters stand for three domains of phototropin. The big Cs in the LOV domains indicate the cysteine residues that have been shown to form an adduct with FMN in Phot 1 of *Avena sativa.* The sequence within the kinase domain marked in italics has not been ovserved in other phototropins. The deduced amino acid sequence of the *C. reinhardtii* phototropin was compared with those from *Oryza sativa (PHOT1)*, acc. AB018443 *Oryza sativa (PHOT2)*, acc. AB018444 *Arabidopsis thaliana (PHOT1)*, acc. AF030864 *Arabidopsis thaliana (PHOT2)*, acc AF053941 *Avena sativa (PHOT1a)*, acc. AF033096 *Avena sativa (PHOT1b)*, acc. AF033097 *Zea mays (PHOT1)*, acc AF033263 *Pisum sativum (PHOT1)*, acc. U83281 *Adiantum capillusveneris (PHY3)*, acc AB012082 *Adiantum capillus-veneris (PHOT1)*, acc. AB037188. An asterix indicates completely conserved residues, a colon indicates a conserved residue.

As shown in Figure 2.2.2, *Chlamydomonas reinhardtii* is heterothallic and isogamous. The mating type (mt+ or mt-) is permanently determined in a cell line and behaves as a single Mendelian locus in crosses. The gametes of mt+ and mt- are similar in size and appearance but different in ultra structural level.

Gametogenesis always appears when adverse growing conditions are provided. Two steps are necessary for gametogenesis. The first one required is nitrogen deprivation (Sager and Granick, 1954). Gametes are quite different from vegetative cells with respect to biochemistry, sub cellular morphology and behavior. Protein synthesis continues even in fully differentiated gametes (Jones et al., 1968; Jones and Chen, 1970) although net protein synthesis ceases shortly after nitrogen deprivation (Jones et al., 1968). Both cytoplasmic ribosomes and chloroplast ribosomes are degraded (Siersma and Chiang, 1971; Martin and Goodenough, 1975; Martin et al., 1976). New ribosomes, which have different sensitivity to antibiotics and ribosomal proteins with altered electrophoretic mobility, are synthesized. The degradation of old ribosomes provides the nitrogen source for *de novo* synthesis of proteins and nucleic acids which are essential for gametes (Jones,

1970; Siersma and Chiang, 1971). Two new organelles appear in gametes, one is a mating structure and the other is a special type of Golgi-derived vesicle (Friedman et al. 1968; Martin and Goodenough, 1975).

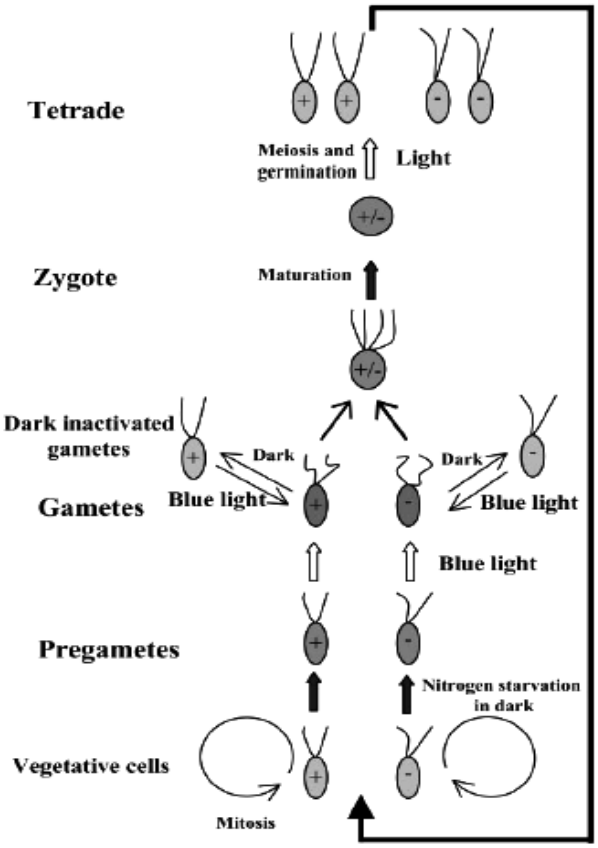


Figure 2.2.2 Life cycle of *Chlamydomonas reinhardtii* (Huang et al., 2003)
In the life cycle of *C. reinhardtii*, light is the essential factor in two step of the growth and development. The first step is the swich from pregamete to gamete and the second step is zygote germination.

After removal of the nitrogen source, vegetative cells become pregametes, which are still not mating competent. Different factors have been tested and light has been shown as required for gametogenesis (Sager and Granick, 1954; Kates and Jones, 1964). Light was defined as a necessary factor for gametogenesis by the work of Christoph F. Beck's group (Treier et al., 1989). DCMU, an inhibitor for photosystem II, was found to be able to prevent gametic differentiation in acetate-free medium but had no effect when acetate was present. Since acetate was used for growing vegetative cell in darkness, light was thought to be the energy source for gametogenesis (Treier et al., 1989). It was also found that the two signals, nitrogen starvation and light input, should be applied successively in gametogenesis. Treatment with the cytoplasmic protein synthesis inhibitor anisomycin or RNA synthesis inhibitor actinomycin D can stop the switch from pregametes to gametes while these two reagents do not effect the mating efficiency of mature gametes (Treier et al., 1989). Action spectrum for the light-dependent step in gametic differentiation was

taken, and in the range between 321nm and 512nm, two peaks (450nm and 370nm) were found (Weissig and Beck, 1990). This result gives the hint of a potential blue light receptor attending in gametogenesis.

It was also reported that the mating competence was only maintained in the presence of both extrinsic signals: lack of nitrogen source and light. Mature gametes lost their mating competence when they were kept in darkness (Beck and Acker, 1992). Although the light induced switch from pregametes to gametes takes around 2 hours and require cytoplasmic protein synthesis, the conversion from dark-inactivated gametes to gametes is much faster and does not require protein synthesis (Pan et al., 1997). Among three wave lengths (451nm, 573nm, 655nm) tested, blue light was most effective in restoring the mating ability of dark-inactivated gametes. In pregametes, flagellar agglutinin activity was hardly detectable, the titer of flagellar agglutinin rose during pregamete to gamete conversion induced by light and decreased in the following dark treatment. Reillumination of dark-inactivated gametes helped them to regain this ability. While the cell body agglutinin activity remained constant in the process, the activity of the gamete lytic enzyme only raised in the process of pregamete-to-gamete switch and did not change in the dark. Reillumination gave very little increase in activity (Pan et al., 1997). The flagellar agglutinin activity could be the key factor in the switch between gametes and dark-inactivated gametes. Given the localization of phototropin in flagella (Huang et al., 2004), phototropin could have relation with the light-induced regaining of flagellar agglutinin activity.

Like in our experiments, an RNAi construct was made to reduce the phototropin level in *Chlamydomonas reinhardtii*. Compared to wild type cells, the selected Phot1- strain had a lower gamete formation percentage in the light-induced pregametes to gametes switch. Although the Phot1- strain also had a fluence dependent response for the pregametes to gametes conversion, the percentage of competent gametes was much lower compared to wild type strain (Huang et al., 2003). The *GLE* (encoding gametic lytic enzyme) mRNA levels increased when wild type pregametes were irradiated. In the Phot1- strain, such an increase was not observed (Huang et al., 2003). Reillumination of dark-inactivated gametes with low intensity of light gave a clear difference in the recovery of mating competence of wild type cells and Phot1- cells, while high intensity of light could accelerate the changing in Phot1- strain (Huang et al., 2003).

It has been shown that zygote germination was controlled by light (Gleockner and Beck, 1995). Under the same duration of illumination, zygotes produced from mating of wild type cells had a higher zygote germination than zygotes that have one Phot1- parental side. Zygotes which had both Phot1- parental strains had the lowest zygote germination (Huang et al., 2003). Since those zygotes were produced from Phot1- gametes, it has not been decided that the failure to germinate was caused by less amount of phototropin in zygote or the disability of germination caused by less amount of phototropin in gametes.

Among the different stages of *C. reinhardtii* life cycle, non-dividing vegetative cells, pregametes and mature gametes are motile. Vegetative cells are attracted to the preferred nitrogen source--ammonium (Sjoblad and Frederikse, 1981; Ermilova, 1993; Ermilova et al., 2000). However, mature gametes do not have chemotaxis to ammonium. The change of chemotaxis is closely related to the switch between pregametes and mature gametes. Nitrogen deprivation and light are the two sequential input signal required for the switch (Sager and Granick, 1954; Ermilova et al., 2003a; 2003b). Three mutants (lrg) which can form gametes in darkness (Gleockner and Beck, 1995) also showed independence of light in losing chemotaxis behavior (Ermilova et al., 2003a). The similarity suggested the possibility of sharing common components in the signal transduction of the two process (Ermilova et al., 2004). The minimal time for nitrogen starvation was around 4 hours before light illumination was applied to induce loss of chemotaxis. It was found that the reduction of fluence resulted in slower kinetics for the loss of chemotaxis activity. Among four wave lengths with identical fluence

rates tested (blue (459 nm), green (540 nm), yellow (573 nm) and red (655 nm)), only blue light was effective in inducing the loss of chemotaxis (Ermilova et al., 2004). Phot1- strains needed longer light illumination after nitrogen starvation to lose chemotaxis. For Phot1- strains, re-illumination of dark-inactivated gamete to turn off chemotaxis also took longer time than in wild type cells (Ermilova et al., 2004).

2.3 *Chlamydomonas reinhardtii* as host for recombinant protein expression

Chlamydomonas reinhardtii, a unicellular organism, is attracting more and more attention these years and is regarded as a green yeast (Goodenough, 1992; Rochaix, 1995). Compared with other known expression systems, *Chlamydomanas* cells many beneficial characteristics. 1.) Nuclear transformation and chloroplast transformation of *Chlamydomonas* are reliable and easy to carry out. Unlike higher plants, the time between the initial transformation and final scale up for production is comparatively short. 2.) *Chlamydomonas* can form gametes, the process of gametogenesis is easy to handle. Genetic crosses between different strains of opposite mating type can be carried out and vegetative diploid cells can be produced. 3.) *Chlamydomonas* can be grown either phototrophically in synchronized or unsynchronized way, or it could be also grown in darkness with acetate as carbon source. 4.) The cost of growing Chlamydomonas is cheap and to grow big volume such as 500,000 L is affordable. 5.) Foreign proteins tend to be folded correctly in *Chlamydomonas* (Mayfeild and Franklin, 2005).

Chlamydomonas reinhardtii has a single, cup-shaped large chloroplast which counts for 40% of the cell volume. This chloroplast contains its own genome, which is a 196kb circular genome. One chloroplast contains 80 identical copies of this chromosome. The first successful chloroplast transformation was reported in 1988 (Boynton et al., 1988), in which *Chlamydomonas* was transformed by DNA coated tungsten particles with a particle gun. Integration of the transforming DNA takes place by homologous recombination. Screening of chloroplast transformant is carried out either by co-transformation with DNA conferring resistance to antibiotics (Goldschmidt-Clermont and Rahire, 1991; Fischer et al., 1996), or by transformation with DNA which helps to rescue phototrophy (Boynton et al., 1988). Chloroplast gene disruption could be either homoplasmic or heteroplasmic. Homoplasmic disruption requires that all 80 copies of chloroplast genome are changed by transformation while heteroplasic disruption means that only some copies of chloroplast genome are changed. Obviously stable transformation requires all 80 copies converted to recombinant form. The latter case happens for gene with essential functions (Rochaix, 1995).

However, the way leading to overexpression of foreign genes in *Chlamydomonas* chloroplast was not smooth at the beginning. Early work in *Chlamydomonas* chloroplast transformations mainly focused on complementation of mutants deficient in photosynthetic genes (Boynton et al., 1988), and on the expression

of proteins with resistance to antibiotics (Goldschmidt-Clermont and Rahire, 1991; Fischer et al., 1996). Trials to overexpress common reporter gene such as β-glucuronidase (GSU) and *Renilla* luciferase result in low level of protein expressions (Savaldor et al., 1993a; Savaldor et al., 1993b; Ishikura et al., 1999; Minko et al., 1999). The reason was later found to be caused by the strong codon bias of *Chlamydomonas* chloroplast, the possibility of A or T appearing in the third position is around 80% (Mayfield and Franklin, 2005). The chloroplast codon optimized GFP gene transformant could increased expression level up to 80-folds compared with its non-optimized counterpart. And when driven by *rbcL* promoter and 5'UTR, it counts for 0.5% of total soluble protein in chloroplast (Franklin et al., 2002). However, independent homoplasmic transformants could have different level up to 5 folds, which could either be caused by mutagenic effect of random integration during transformation or by 5-fluoro-2-deoxyuridine (FdUdr) which is used to decrease chloroplast genome number prior to transformation (Mayfield et al., 2003). Recently, high level of expression and assembly of a human monoclonal antibody in *Chlamydomonas reinhardtii* was reported (Mayfield et al., 2003), which suggest the capability of *Chlamydomonas* chloroplast in expressing complex biomolecules in its active form.

Compared with overexpression of recombinant protein in *Chlamydomonas* chloroplast, over-expression based on nuclear transformation seems more difficult and meets lots of hindlerers although expression of foreign DNA sequences in *Chlamydomonas* was first reported in 1982 (Rochaix and van Dillewijn, 1982). They managed to express yeast *ARG4* gene in *cw-15 arg*-7, and it was six years earlier than the first report of *Chlamydomonas* chloroplast transformation. For nuclear transformation, several well established methods are available. Cell wall deficient strains can be transformed with glass bead method readily (Kindle, 1990). Walled strains can either be treated with autolysin to shed the cell wall and then the glass bead method is applied, or silicon carbide can be used in place of glass bead for walled cell transformation (Dunahay 1993). Particle guns have also been used for transforming wild type *Chlamydomonas* cells (Debuchy et al., 1989; Kindle et al., 1989; Mayfield et al., 1990).

At the beginning, the selectable markers used for most studies are *Chlamydomonas* genes which are capable of complementing mutants to wild type phenotype. The genes employed are *ARG7* (Debuchy et al., 1989), *NIT1* (Kindle et al., 1989), *OEE1* (Mayfield et al., 1990), *atpC* (Smart and Selman, 1993), *NIC-7* (Ferris, 1995) and *THI-10* (Ferris, 1995). Recently genes which confer *Chlamydomonas* resistance against antibiotics are widely used for cotransformation, such as *CRY1* (Nelson et al., 1994), *Ble* (Stevens et al., 1996), *APH-VIII* (Sizova et al., 2001), *APH-7* (Berthold et al., 2002).

However, the use of *Chlamydomonas* to express foreign genes has met great difficulties and there are only few successes with several reporter genes which are routinely used in other eukaryotic systems (Day et al., 1990; Blankenship and Kindle, 1992; Kindle and Sodeinde, 1994; Sizova et al. 1996; Stevens et al., 1996). The reason for such phenomenon could be certain characteristics of *Chlamydomonas*. During transformation, foreigh DNA randomly integrated into *Chlamydomanas* genome (Rochaix, 1995). Only those DNA fragments integrated into transcriptional active regions would get good expression. Similar to its chloroplast genome, *Chlamydomonas* nuclear genome also has a strong codon bias. It has high GC content (62%) and prefers to have G or C in the third position of the codon (Harris, 1989; Silflow, 1998). A codon optimized GFP gene got nice expression after transformation (Fuhrmannet al., 1999) in *Chlamydomonas*.

Another feature of *Chlamydomonas* gene is that most contain introns (Harris, 1989). It is possible to rescue metabolic deficient mutants with cDNA while the expression levels of cDNA are much lower than their genomic counterparts (Diener et al., 1993; Auchincloss et al., 1999; Perron et al., 1999; Boudreau et al.,

2000). It was suggested that introns could take part in the regulation of transcription and it was also shown that insertion of the first intron of *rbcS2* gene could increase the strength of *rbcS2* promoter up to 30 folds. Intron 1 of *rbcS2* gene functions as an enhancer and its enhancer function is independent of its position and orientation (Lumbreras, 1998).

Another problem of nuclear expression is that *Chlamydomonas* does not have very strong promoters. The *rbcS2* promoter has been widely used and is regarded as the strongest promoter for heterologous gene expression (Goldschmidt-Clermont and Rahire, 1986; Berthold et al., 2002; Fuhrmann et al., 1999). Recently it was found that the strength of the *rbcS2* promoter could be improved by placing the *HSP70A* promoter in front of it (Schroda et al., 2000). Thus the *HSP70A* promoter combined with *rbcS2* promoter plus *rbcS2* intron 1 is currently the most powerful promoter available for expression in *Chlamydomonas.*

The *PsaD* gene encodes a 20kD subunit of photosystem I (Zilber and Malkin, 1988; Chitnis et al., 1989). This gene contains no introns, which suggest that all the regulatory sequences leading to high expression are located in the flanking regions. The expression of the *ble* gene driven by the *PsaD* promoter is almost similar to the expression driven by *HSP70A* plus *rbcS2* promoter with *rbcS2* intron1. It is regarded as a very powerful tool in expressing recombinant cDNA in *Chlamydomonas* nucleus (Fischer and Rochaix, 2001).

2.4 RNA interference and gene silencing

RNAi (RNA interference) is a powerful tool for gene silencing in all model systems currently studied by biochemists. Fire et al., (1998) discovered it while investigating the use of antisense and sense RNA for gene inhibition in the nematode worm *Caenorhabditis elegans*. Later, RNAi was found to be a universal defense mechanism for eukaryotic cells against RNA viruses or proliferation of transposable elements that replicate via RNA intermediates. In a wide variety of organisms, such as fungi, plants, worms, mice and probably humans, RNA interference can be applied as useful technique to turn off the expression of individual cellular genes.

The most important factor in RNA interference is a so called short interfering RNA (siRNA). siRNAs are 21-23nt dsRNA duplexes with symmetric 2-3 nt 3'overhangs. The 5'end of siRNA normally carries a phosphate, while 3'end does not. siRNAs are created by Dicer, a member of RNase III family. Dicer uses the energy of hydrolysis of ATP and digests dsRNA into siRNA (Bernstein et al., 2000; Ketting et al., 2001; Billy et al., 2001). This process takes place in cytoplasm (Hutvagner and Zamore, 2002; Zeng and Cullen, 2002; Kawasaki and Taira, 2003).

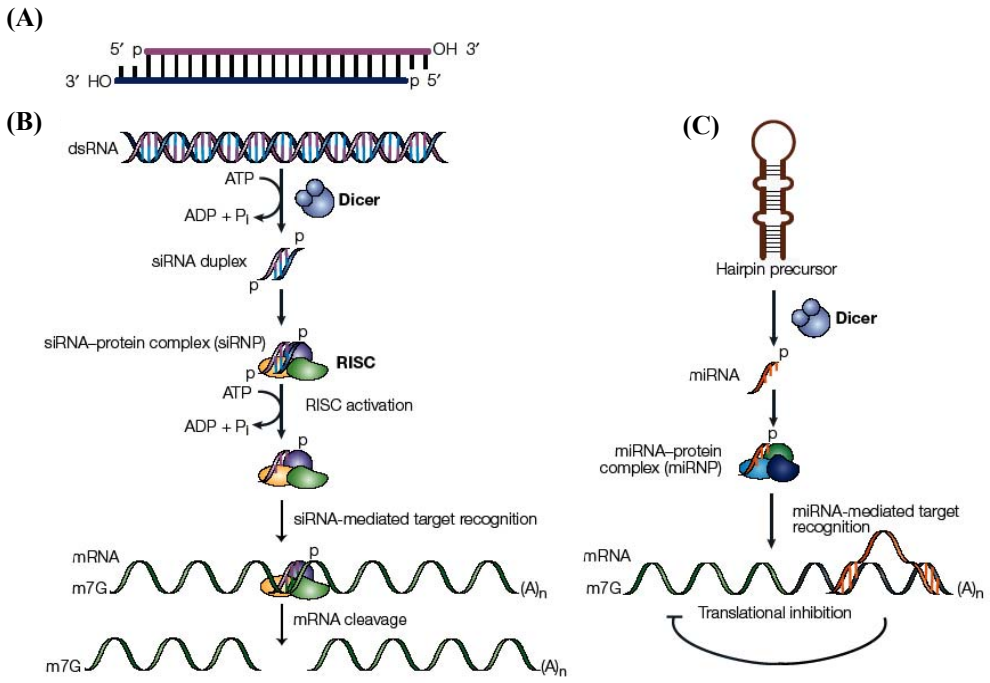


Figure 2.4.1 The RNAi pathway. (Dykxhoorn et al 2003)

(A) Short interfering RNA (siRNA). An siRNA is composed of two 21-nt ssRNA with a 19-nt duplex region. The 5'-ends of both strand are phosphorylated. (B) The siRNA pathway. Long double-stranded RNA (dsRNA) is digested by Dicer in an ATP-dependant way. Then siRNAs are uptaken by RISC. ATPs are hydrolysed to help unwind of siRNA but the incorporation is ATP independant. The single-stranded antisense strand help RISC to find the target mRNA, and the mRNA is cleaved in the middle of the duplex region, 10 nt from the 5'end of the siRNA. (C) The micro (mi) RNA pathway. Dicer also cleave the ~70-nt imperfect hairpin RNA and produce ~22nt miRNA, those miRNA would bind to the 3'untraslated region (UTR) of their target mRNA and block translation.

siRNAs then bind to a protein complex called RISC (RNA-inducing silencing complex). 5'-phosphate of siRNA is strictly required for binding and those siRNA which lack 5'-phosphate will be quickly phosphorylated and incorporated into RISC (Nykanen et al., 2001; Schwarz et al., 2002). The two strands of siRNA separate and one strand continues binding to RISC. The single-stranded RNA which remains in the complex works as a guide to its homologous target mRNA for endonucleolytic cleavage. RISC will cut mRNA the in the center of the duplex region formed between the guide siRNA and target mRNA, thus the mRNA is degraded (Elbashir et al., 2001).

In mammals, endogenously expressed siRNA has not been discovered but a similar miRNA (micro-interfering RNA) pathway has been found in many organisms and cell types (Pasquinelli, 2002). miRNA is also generated by Dicer, but in contrast to miRNA, those short RNA species (~22nt) are produced from precursors about ~70nt with imperfect hairpin. They are single-stranded. miRNAs will recognize homologous regions in target mRNA and thus prevent translation.

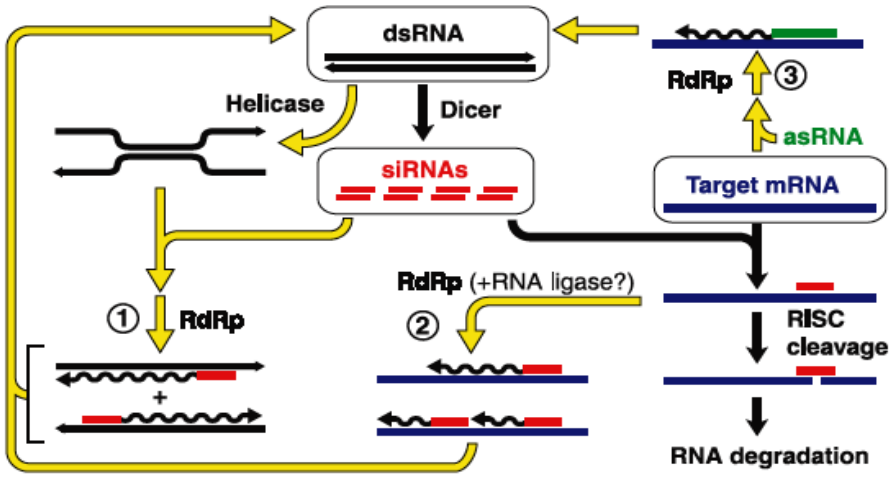
Many viruses have RNA dependent RNA polymerase (RdRps) to replicate their RNA genomes. Many eukaryotes also encode RdRps and use them in a sequence specific, RNA-triggered gene silencing mechanism (Ahlquist, 2002). RdRps make RNAi a self-amplifying process. RdRps can use single-stranded

siRNA as primer and target mRNA, degraded target mRNA or melted dsRNA as template to form new dsRNA. These dsRNAs would enter the siRNA pathway again.

There are many different ways to trigger the RNA interfering process. The way to generate RNAi can be divided into two major groups, one is to use RNAs that are prepared *in vitro*, the other is to use RNA which is generated *in vivo*. The first group includes using chemically synthesized siRNA, using long dsRNA, using siRNA-based hairpin RNA or using miRNA-based hairpin RNA. The RNAs would be injected into the host to silence a specific gene. The RNAs prepared *in vitro* only last for a limited period of time. The second group is based on transforming the host with genes that would form double-stranded RNA or hairpin RNA after transcription. Promoter either for RNA polymerase II or for RNA polymerase III are used, the sense and anti-sense chains are either under the control of same promoter or arranged in a tandem way and the two single-stranded RNAs will form pairs (Dykxhoorn et al., 2003).

In *Chlamydomonas*, the AR promoter (*HSP70A* plus *rbcS2*) is used preferentially to get strong transcription. A genomic piece of target gene is taken and the reverse cDNA counterpart is placed behind. A 3'UTR is placed behind the RNAi construct in some cases (Huang et al., 2002), but construct without 3'UTR also seems to work fine (Fuhrmann et al., 2001).

(A)



(B)

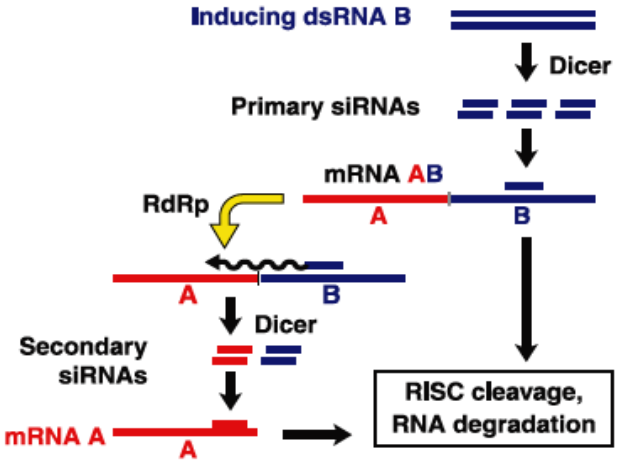
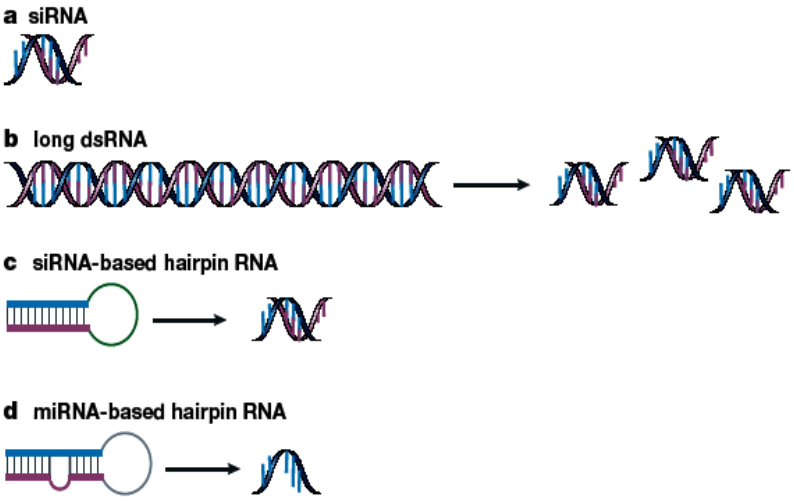


Figure 2.4.2 Self-amplifying process of RNAi and transitive RNA siliencing (Ahlquist, 2002).

(A) Degradative and synthetic pathway connecting dsRNA, siRNA and mRNA. The black arrows outline the classical degradative pathways in RNAi process and yellow arrows denote the synthetic pathways. RdRps may act on siRNA-primed dsRNA(1), siRNA-primed mRNA(2) or asRNA-primed mRNA. The product, dsRNA, will re-enter the RNAi process to generate more siRNA. (B) Transitive RNA silencing. When there is intermediary mRNA AB or another mRNA which contains high homologous region of gene B, the RNAi reaction which targeted gene B would also cause the silencing of gene A.

(A)



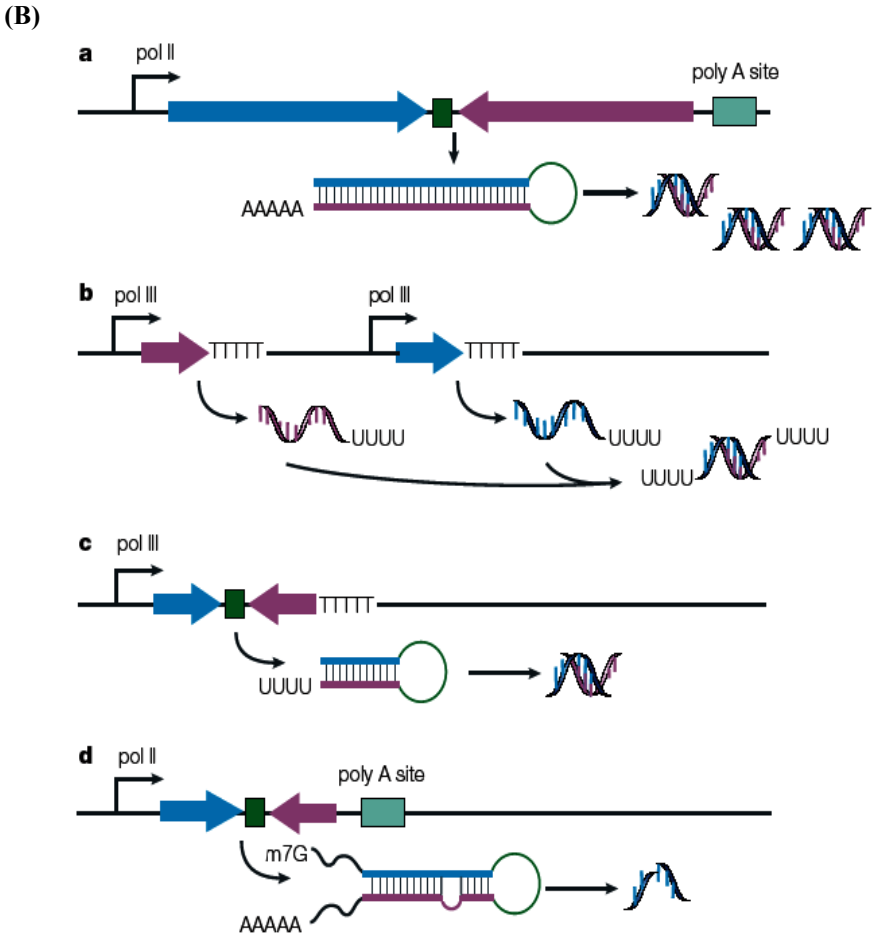


Figure 2.4.3 Methods to generate RNAs that silence gene expression (Dykxhoorn et al., 2003).

(A) Silencing by RNAs generated *in vitro*. Aa, Chemically synthesized siRNA which could function in absence of Dicer. Ab, Long dsRNA enters the RNAi process directly. Ac, Perfect duplex hairpin RNA induces siRNA pathway. Ad, Imperfect duplex hairpin RNA induces miRNA pathway. (B) Silencing by RNAs generated in vivo. Ba, RNA polymerase II promoter was used and perfectly symmetric sense and anti-sense chains are under the control of same promoter. Long hairpin RNA will form after transcription. Bb, Sense and anti-sense strands are under the control of tandem RNA polymerase III promoter. Bc, Single RNA polymerase III promoter is used to control the transcription of siRNA-based hairpin RNA. Bd, Single RNA polymerase II promoter is used to control the transcription of the miRNA-based hairpin RNA.

2.5 The goal of *C. reinhardtii* mating assay

As described in Chapter 2.2, light, especially blue light, is of great importance in the sexual life of *C. reinhardtii*. But, it is impossible to study the involvement of phototropin in zygote germination separately from gametogenesis and maintenance of mating competence in the way described in Huang et al., (2003).

The common way to study zygote germination is very complicated and few labs are able to carry it out. First, gametes of both mating types should be generated according to the standard protocols. Then the gametes

are mixed and mate. After two hours, the cells are diluted and plated on TAP plates with 4% agar and those plates are illuminated for 24 hours and then transferred to the dark for 5 days. The vegetative cells and unmated gametes are then removed with a razor blade, leaving the hard-walled zygospores adherent to the agar. The plates are then treated with chloroform vapor for 30 seconds (Harris, 1989). To study the relationship between light and zygote germination, those two steps should be carried in darkness. Then the plates are placed under light for 1-5 hours and transferred into darkness again. 11 days later, the numbers of germinated and non-germinated zygotes are counted under a dissecting microscope (Huang et al., 2003). There are several drawbacks of this method. 1.) The procedure is difficult to handle. 2.) Since there are many steps, manipulation on each plate can hardly be identical, which may cause errors in the final result. 3.)Vegetative cells and unmated gametes can hardly be removed totally. 4.) This method assumes that the only difference between zygotes produced from wild type cells and the zygotes produced from Phot1- strains was the level of phototropin. Given the involvement of phototropin in gametogenesis and maintenance of mating competence, this point was questionable.

Thus, a new mating assay was required to study phototropin and zygote germination. The test should be easily carried out, involve less steps and be able to distinguish zygotes from vegetative and unmated gamete more efficiently. In this thesis, a new mating assay was established which fulfilled all requirements.

3. Results

3.1 Localization and expression profile of Phototropin

3.1.1 Distribution and localization of phototropin

As a temporarily membrane associated protein, phototropin in *Chlamydomonas* exists in the membrane fraction, the soluble fraction and the flagella fraction of the whole cell lysate. In different strains, the distribution of phototropin in membrane and soluble fraction varies. In different growth stages, the phototropin level and distribution varies.

Figure 3.1.1.1 Localization of phototropin

(A) Western blot analysis of the localization of phototropin in vegetatively grown wild type strain *CC125mt(+)*. Cells were fractionated into flagella fraction, soluble fraction and membrane fraction. Rabbit antiserum raised against *E. coli* expressed *Chlamydomonas* Phototropin LOV1 domain was used. Lane F: flagella fraction of *CC125mt(+),* Lane S: soluble fraction of *CC125mt(+)* after deflagellation, Lane M: membrane fraction of *CC125mt(+)* after deflagellation. Cells were harvested at exponential stage. 80µg of protein was loaded in each lane. (B) Western blot analysis of the localization of phototropin in flagella. Flagella prepared from *CC125mt(+)* vegetative cells by pH shock protocol were fractionated into soluble fraction, insoluble fraction. Same antiserum as in (A) was used. Lane FS: Soluble fraction of *Chlamydomonas* flagella. Lane MA: Insoluble protein of *Chlamydomonas* flagella. Phot1, full-length phototropin; Phot1 D1, degraded phototropin.

In order to find out the common distribution pattern of phototropin in membrane, soluble fraction and flagella, the wild type strain *CC125mt(+)* was used (Figure 3.1.1.1). Different from what was reported by Huang et al., (2001), in which phototropin was described to exist only in membrane attached form in cell body, it has been shown that phototropin also exists as a soluble protein in *C. reinhardtii.* Comparison between flagella fraction (F), membrane fraction (M) and soluble fraction (S) showed that phototropin concentration is highest in flagella. Compared with the membrane attached form, phototropin in soluble form has a smaller molecular weight (~2kD) (Figure 3.1.1.1 A), suggesting that the soluble form is probably inactive.

Isolated *Chlamydomonas* flagella was broken by freeze/thaw treatment and carefully fractionated into soluble fraction and insoluble fraction. It was found that in flagella, phototropin also existed both in soluble form and insoluble form. The soluble phototropin of flagella also had a smaller molecular weight (~2 kD) compared with the insoluble form (Figure 3.1.1.1 B). The degraded phototropin also appeared in flagella fraction. It only

remained insoluble in flagella.

In western blots, two bands (~80kD band and ~60kD band) always appeared which could be detected by rabbit antiserum raised against phototropin LOV1 domain. Whether there is a second phototropin has been a puzzle in this study for a long time. The lower band seems to appear only in the membrane fraction. In the *Chlamydomonas* genome project, it was proved that there was only one phototropin in *Chlamydomonas* genome. The 60kD band was assumed to be a degradation product of phototropin . Since an antibody raised against *Chlamydomonas* Phot1 kinase domain only recognized the 80kD band (Huang et al., 2002; 2004), the lower band is regarded as a C-terminal degradation product from Phototropin.

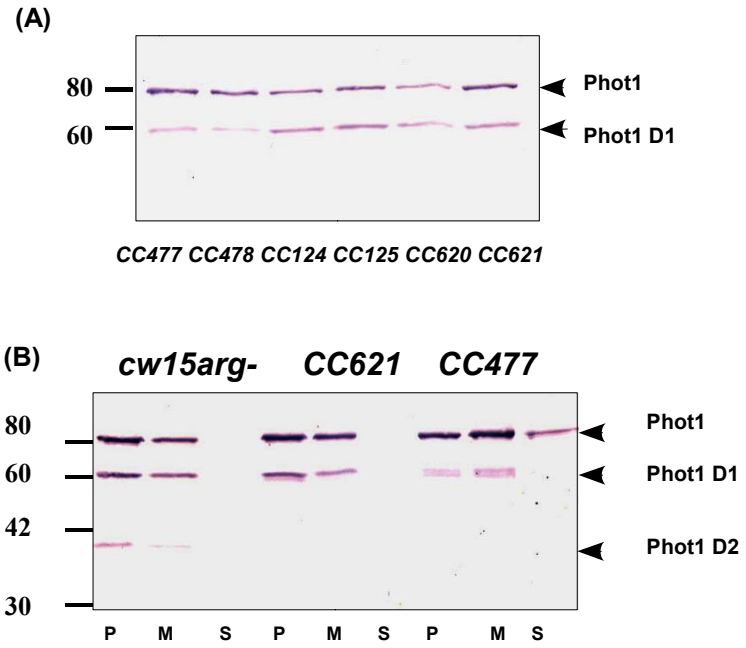


Figure 3.1.1.2 Phototropin level and distribution in different strains grown under same conditions

(A) Western blot result of Phototropin levels in different strains grown under same coditions. Strain *CC477, CC478, CC124, CC125, CC620, CC621* were tested. Same amount of cell lysate (80µg) was loaded in each lane. Rabbit antiserum against phototropin LOV1 domain was used. (B) Western blot result of the distribution of phototropin in different strains. Vegetatively grown cells were fractionated into three fractions: low speed pellet (P) (13,000×15min), membrane fraction (M) (120,000g×40min) and soluble fraction (S). Strain *cw15 arg-*, *CC621* and *CC477* were tested. 80g protein of each fraction was loaded in each lane. Same antiserum as in (A) was used. Phot1, full-length phototropin; Phot1 D1, degraded phototropin (~60kD); Phot1 D2, degraded phototropin (~35kD).

In different strains, the level and localization of Phot1 differs. As shown in Figure 3.1.1.2 (A), six different strains (*CC477, CC478, CC124, CC125, CC620, CC621*) grown under same conditions (4W/m^2, 120rpm, 25°C) were tested. Although the same protein amount of cell lysate was loaded in each lane, the phototropin amount seemed to be different in each analysed fraction. That suggests that the phototropin level is delicately regulated. Even under identical growing conditions, slight difference of strains cause a difference in phototropin levels.

It was also discovered that the distribution of phototropin in different tested strains was different. To illustrate the regulation of phototropin distribution, three strains with special characters were picked. *cw 15 arg- A* is a cell wall deficient strain with very thin and broken piece of cell wall attaching to cell membrane. *CC621* is a wild type strain and *CC477* is a bald strain and has no flagella to swim. Those strains were grown under identical condition (4W/m^2, 120rpm, 25°C) and fractionated as described by Huang et al., (2002). In those

strains, the distribution of Phototropin in low speed pellet (13,000g×5min), membrane fraction (120,000g×40min) and soluble fraction appears different. The soluble portion of phototropin was more obvious in the bald strain *CC477* compared with other two strains. In the cell wall deficient strain *cw15 arg- A*, almost all the phototropin is found in the membrane attached form. A third band which around 35 kD was detected in this strain. It is still unknown whether this band results from phototropin. It did not appear in the fusion overexpression experiment results in *C. reinhardtii* although it did appear in the oocyte expression results (see chapter 3.4). The ratios between full length phototropin and degraded version of phototropin's distribution in membrane fraction and in low speed pellet also varied in those three strains.

3.1.2 Distributions of phototropin in different growth stages

Wild type strain *CC621* and *CC620* were chosen in this experiment since their mating competences are the best among currently known strains, the gametogenesis rate can reach 100% (Harris, 1989).

From the western blot results (Figure 3.1.2.1), it was concluded that phototropin occurs in low speed pellet, membrane fraction and soluble fraction of all stages of vegetatively growing cells and gametes. In the early stage of vegetative cell, very small amounts of soluble phototropin was found and most phototropin was attached to the membrane. Soluble phototropin also showed several different smaller molecular weights, which could be caused by degradation induced by strong illumination. As the cells reached the stationary stage, more phototropin remains soluble since high cell density provided each cell with a comparatively darker surrounding, which prevented phototropin getting 'activated'. Some unknown bands were detected in soluble fraction, which has smaller molecular weight than phototropin (~5-10kD). The identity of the protein is to be discovered. After gametogenesis, more phototropin existed as soluble protein, and there was only one band that was detected in the soluble fraction of both mating type gametes. In all three stages tested, the second band (~60kD), which was regarded as a C-terminal degradation product, remained in the low speed pellet and the membrane fraction. The pattern has only been tested for these two strains. As shown before, the distribution is different in different strains.

Vegetatively grown cells in light and darkness were fractionated (Figure 3.1.2.1). Surprisingly, it was discovered that not only the distribution of phototropin in membrane fraction and soluble fraction, but also the ratio between full length phototropin and truncated phototropin were different. In cells grown in light, less phototropin was soluble. In cells which were grown in darkness, more phototropin remained full length. Light seems to be the cause of phototropin degradation. To test this assumption, experiments about light-induced phototropin degradation were carried out (Chapter 3.1.3).

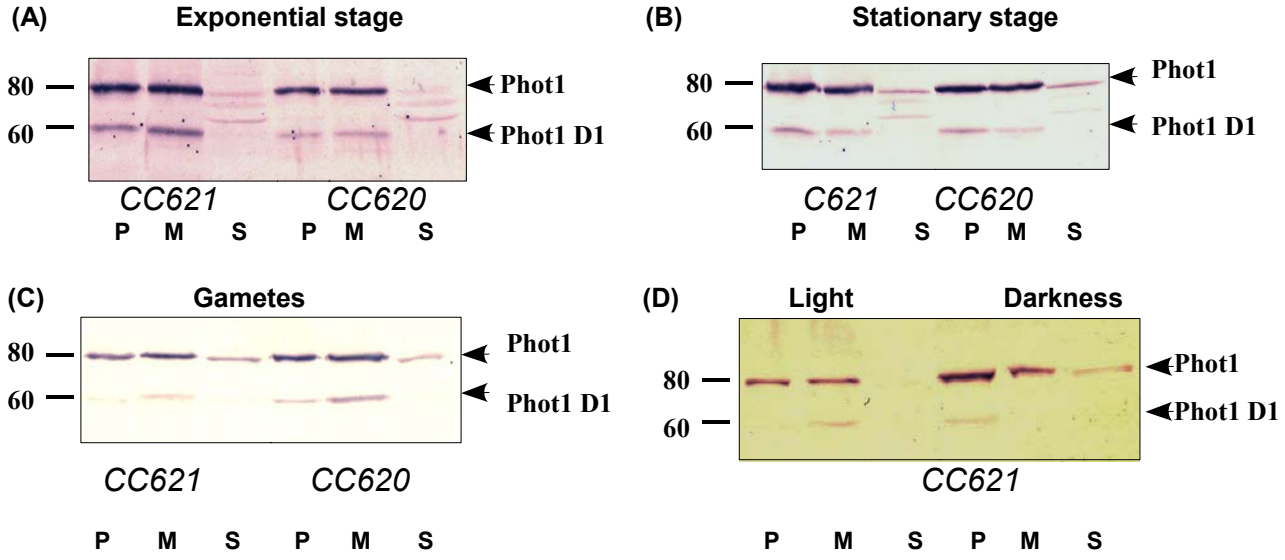


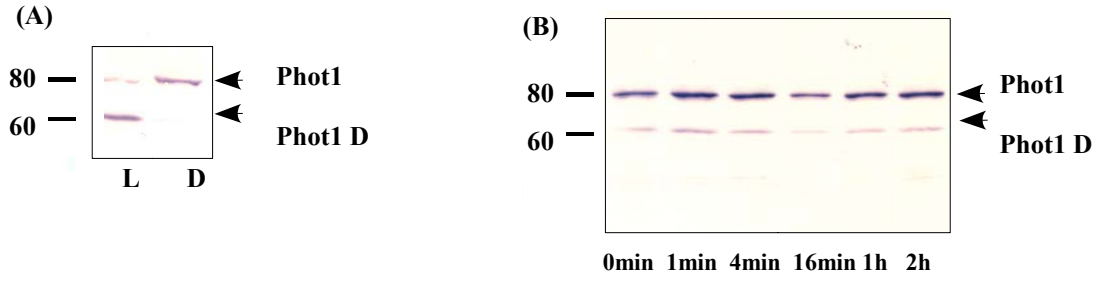
Figure 3.1.2.1 Phototropin distributions in pellet, membrane and soluable fraction in different cell stages and in different light conditions.

(A) Western blot result of the distribution of phototropin in low speed pellet (13,000g×15min), membrane (120,000g×40min) and soluble fraction in the exponential stage of vegetatively growing strains *CC621* and *CC620*. (B) Western blot result of the distribution of phototropin in pellet, membrane and soluble fraction in stationary stage of vegetatively growing strains *CC621* and *CC620*. (C) Western blot result of The distribution of phototropin in pellet, membrane and soluble fraction of gametes of strains *CC621* and *CC620*. (D) Western blot result of the distribution of phototropin in pellet, membrane and soluble fraction of *CC621* grown in light or in darkness. Phot1, full-length phototropin (~80kD); Phot1 D, degraded phototropin (~60kD).In the all four western blots, rabbit antiserum against *Chlamydomonas* phot1 LOV1 domain was used.

The common degradation pattern of phototropin exists in both mating types and through almost all the cell cycle of strain *CC620* and *CC621*. It is interesting that the 60kD degradation product seems to be present only in the membrane fraction and low speed pellet. The 35kD band which was observed in *cw15 arg-* was not observed here. But the 35kD phototropin degradation does appear in many cases (data not shown), and it is not caused by light. The reason is still unclear.

3.1.3 The degradation of phototropin is induced by light.

As in other organisms, phototropin has a feed back regulation pattern (Sakamoto and Briggs, 2002). In *Chlamydomonas*, it can be triggered by the light intensity. It was first discovered that there were two bands in the western blot with whole cell lysate which could be detected by antibody raised against phototropin LOV1 domain. But antibody raised against phototropin kinase domain can only recognize the upper band (Huang et al., 2002; 2003; 2004). The lower band was thought to be the C-terminal truncated product of phototropin.



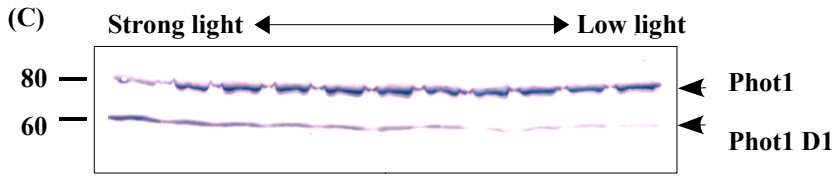


Figure 3.1.3.1 Light induced degradation of phototropin.

(A) Comparison of cells (*CC621*) grown under strong light conditions (9W/m^2) for 2 days and in complete darkness for 5 days. Lane L: whole cell lysate from cells grown in strong light; Lane D: whole cell lysate from cells grown in darkness. 80µg protein is loaded in each lane. (B) Light induced phototropin degradation experiment (Short duration). Cells (*CC48*) grown in complete darkness were harvested in red light and divided into equal amount. High light (9W/m^2) illumination was applied afterwards. Samples were taken after 0min, 1 min, 4min, 16min, 1 hour, 2 hour and same amount of cells was loaded in each lane. No obvious degradation was observed. (C) Light gradient experiment. A light gradient (20W/m^2, 14.2W/m^2, 11W/m^2, 9.4W/m^2, 5.2W/m^2, 3W/m^2, 1.6W/m^2, 0.71W/m^2, 230mW/m^2, 160mW/m^2, 95mW/m^2) was given to cells (*CC124*) for two days. Preculture was grown till its exponential stage under middle light intensity (~4W/m^2) before cells were harvested and inoculated in fresh medium. Same amount of cells were loaded in each lane. Pho1, full-length phototropin; Phot1 D, degraded phototropin. Antibody raised against Phototropin LOV1-domain was used.

It was further found that the ratio of these two bands changes according to light conditions. When cells are grow in darkness, the 80kD band is the main band. And when cells grow under strong light condition (9W/m^2), the 60kD band is the main band (Figure 3.1.3.1(A)). It seems that light intensity determines the ratio of the two bands.

Cells grown in darkness were used for further degradation experiment. The first experiment failed as shown in Figure 3.1.3.1 (B). The degradation process took much longer time than expected. Under strong light illumination, phototropin does not show obvious degradation even after 2 hours. The illumination duration of 24 hours and 36 hours were tried as well, but no significant improvement was found. When the duration was elongated to two days (~48 hours), a clear light induced degradation of phototropin appeared. The full-length phototropin level in cells growing in high light condition was less than that of cells growing in low light condition, while the truncated version of phototropin in high light grown cells was more abundant than that in low light grown cells. Whether the truncated version is just a down regulation of phototropin or it has different function still requires further experiments.

3.2 Reduction of the phototropin level in *C. reinhardtii* by RNAi

3.2.1 The Phot1- RNAi construct

RNAi (RNA interference) is a powerful tool for gene silencing in almost all model systems currently studied by biochemists. In *Chlamydomonas reinhardtii*, RNAi technology was first introduced by Fuhrmann et al., (2001). High transcription efficiency is required to obtain the best result for target gene silencing, thus the strongest promoter currently known was used. This is the *RbcS2* promoter (Goldschmidt-Clermont and Rahire, 1986) combined with *HSP70A* promoter (Schroda et al., 2000) and *RbcS2* intron1 (Lumbreras et al., 1998).

To avoid instability of the plasmid in *E. coli*, which may be caused by palindromic sequence, an artificial genomic piece of *Chlamydomonas Phot1* was used in the RNAi construct. One 836bp fragment from the 5' end of *Chlamydomonas Phot1* cDNA, *RbcS2* intron1 and one short genomic fragment were put together to form the leading genomic sequence. The reason to use *RbcS2* intron was because of its transcriptional enhancer activity (Lumbreras et al., 1998). Behind that, an corresponding inverted cDNA fragment was introduced into the construct. According to Fuhrmann et al., (2001), no 3' UTR was used in the construct.

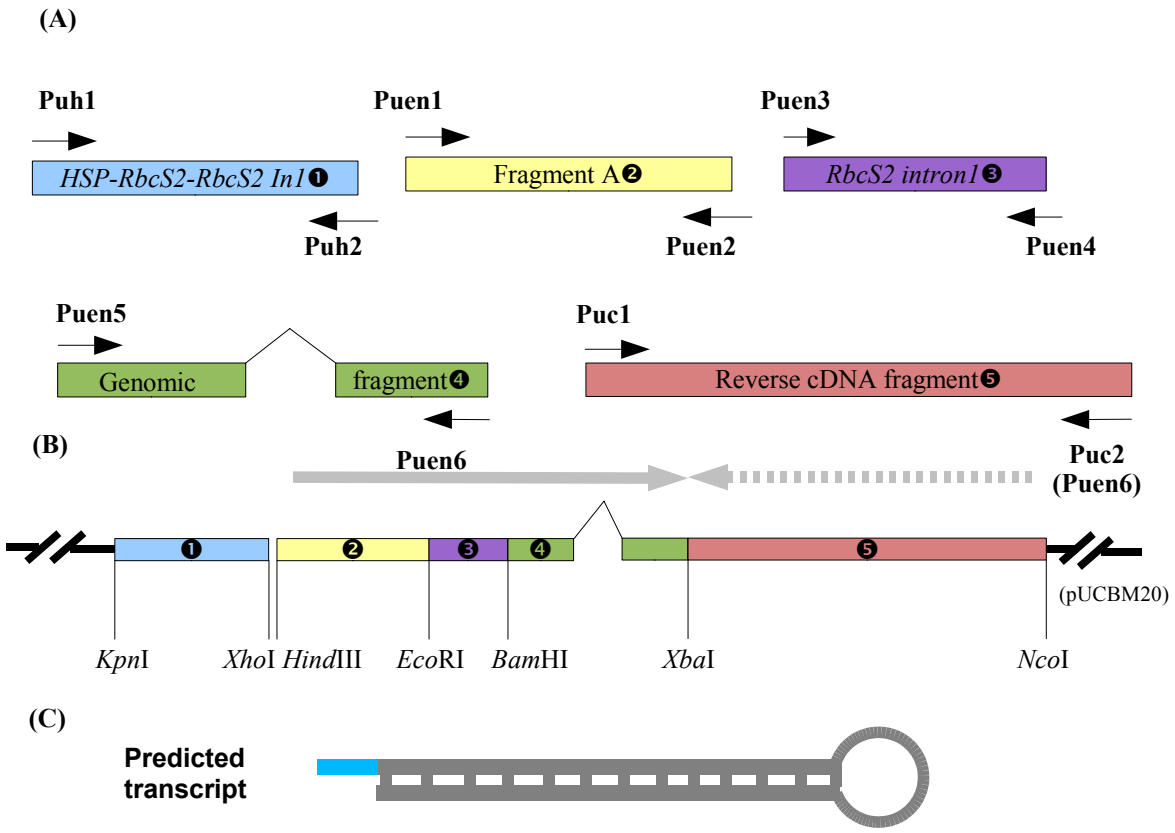


Figure 3.2.1.1 Construction of RNAi construct of *Chlamydomonas Phototropin gene*

(A) Primers used for amplification of different fragment. Those different color boxes represent different fragments. The primers are placed around the fragment which they were used to amplify. (B) Final construct used to transform *Chlamydomonas reinhardtii*. Those color boxes with numbers represent the different fragments same as in (A). The restriction enzyme sites are below the fragments. The orientations of gene segment are indicated by arrows. The vector used is indicated on the right side. (C) Predicted hairpin structure of mature mRNA after post-transcriptional modification. The blue piece stands for promoter region and the rest gray piece stands for the RNAi construct.

The construct would only form a regional hairpin structure close to the joint between the artificial genomic fragment and the inverted cDNA piece, since there were palindromic sequences around the restriction

enzyme site. Because of the two introns (*RbcS2* intron1 and one intron already exist in the genomic piece), the construct should be stable in *E. coli*. After transformation of *Chlamydomonas*, the promoter plus the RNAi construct would integrate into *Chlamydomonas* genome and would be transcribed into mRNA. After splicing, the two introns would be cut out and mature RNA would form a hairpin structure and trigger the silencing process of the target gene.

The construction of the plasmid and the primers are shown in the Figure 3.2.1.

3.2.2 Transformation of *C. reinhardtii* strain *cw15 arg- A* with the RNAi construct

C. reinhardtii strain *cw15 arg- A*, a cell wall deficient strain, was used as a recipient for RNAi construct since it is easy to transform with the glass bead method. pSI103, which contains the *aphVIII* selectable marker gene, was used for co-transformation. Transformants were plated on TAP agar plates containing 50 µg/ml arginine and 15µg/ml paromomycin. A rabbit antibody raised against *Chlamydomonas* Phototropin LOV1 domain was applied to screen RNAi transformants. 25% of paromomycin-resistant transformants showed a reduction of phototropin lower than 40% of the original level and 15% displayed reduction of less than 10% compared to the original level.

Among those transformants obtained, *2A* and *4A* had the lowest level of phototropin. Compared with recipient strain *cw15 arg- A*, the phototropin level was reduced to around 5%. Among 24 clones screened, 5 had obviously decreased phototropin level. Thus it was concluded that the RNAi construct was efficient in silencing phototropin in *cw15 arg- A*.

3.2.3 Transformation of *C. reinhardtii* strain *CC32pab1mt(+)* with phototropin RNAi construct

For the mating assay, a strain was needed which not only could be used for mating process but was also metabolic deficient. *C. reinhardtii* strain *CC32pab1mt(+),* which needs p-aminobenzoic acid for growth*,* was chosen as the candidate for this assay. pSI103 which contains *aphVIII* selection marker gene was used for co-transformation as a marker. Selection of RNAi transformants was performed on TAP agar plates containing 50µg/ml p-aminobenzoic acid and 15µg/ml paromomycin. Rabbit antibody raised against *C.reinhardtii* phototropin LOV1-domain was used for detection of phototropin levels in transformants. Out of more than 1000 transformants with resistance against paromomycin, only one transformant with obviously decreased phototropin level was obtained.

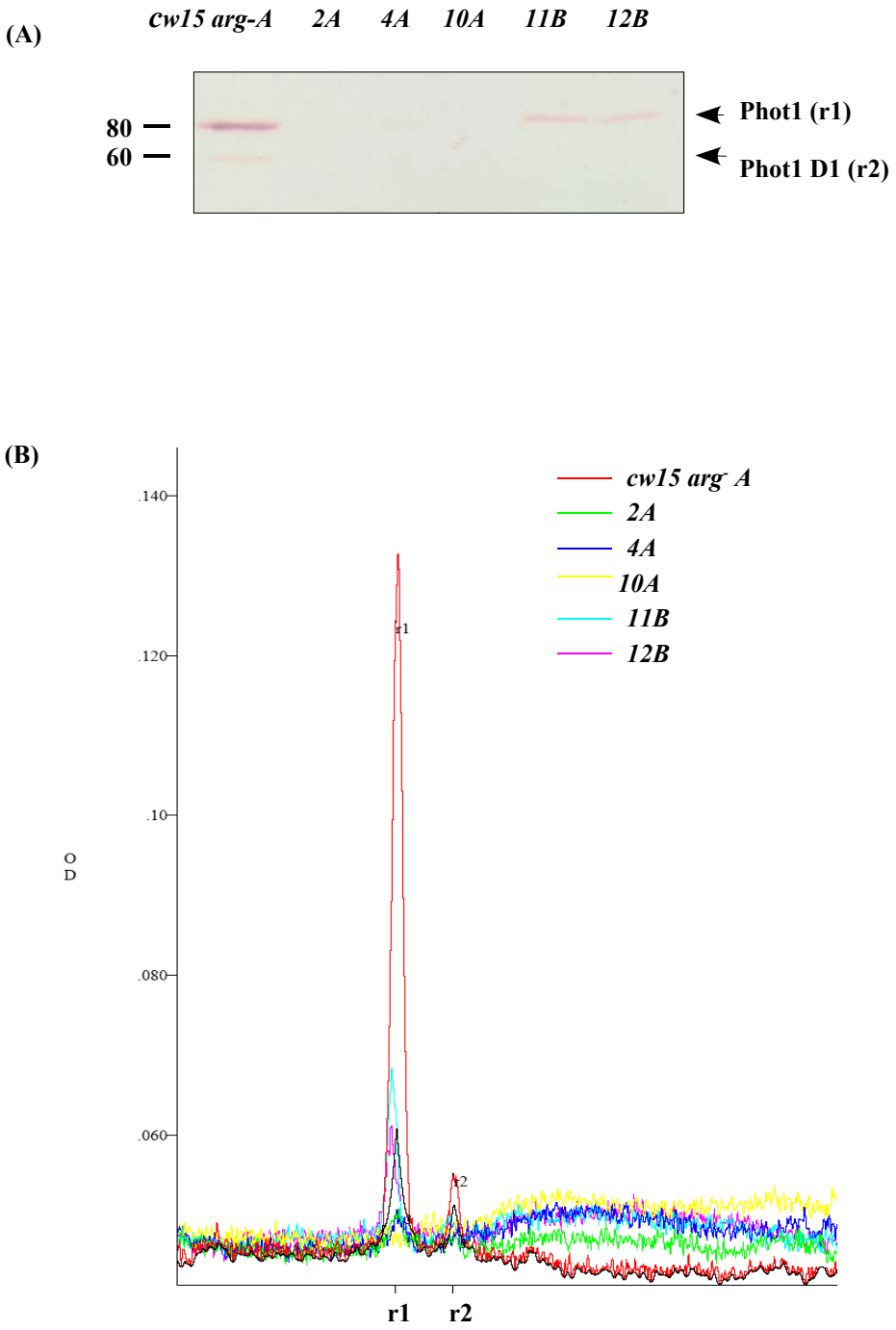


Figure 3.2.2.1 Relative amount of phototropin in the transformants and recipient strain cw15 arg- A

(A) Western blot result of screening. 2A, 4A, 10A, 11B, 12B represent different transformants. Same amount of protein from cell lysate was loaded onto each lane. Antibody raised against *Chlamydomonas* phototropin LOV1 domain was used. (B)Gel analysis result of the western blot. The red line represents the recipient strain cw15 arg- A and other color lines represent those transformants obtained after transformation. The two peaks represent full-length phototropin (r1) and its degraded form (r2). Both proteins level were reduced obviously in all the transformants. OD in the Y-axis represents optical density (Gel-pro analyzer was used to determine the optical density of each band). Phot1, full-length phototropin (80kD); Phot1 D1, degraded phototropin (60kD).

(A) *G5 C4 CC32Pab1mt(+)*

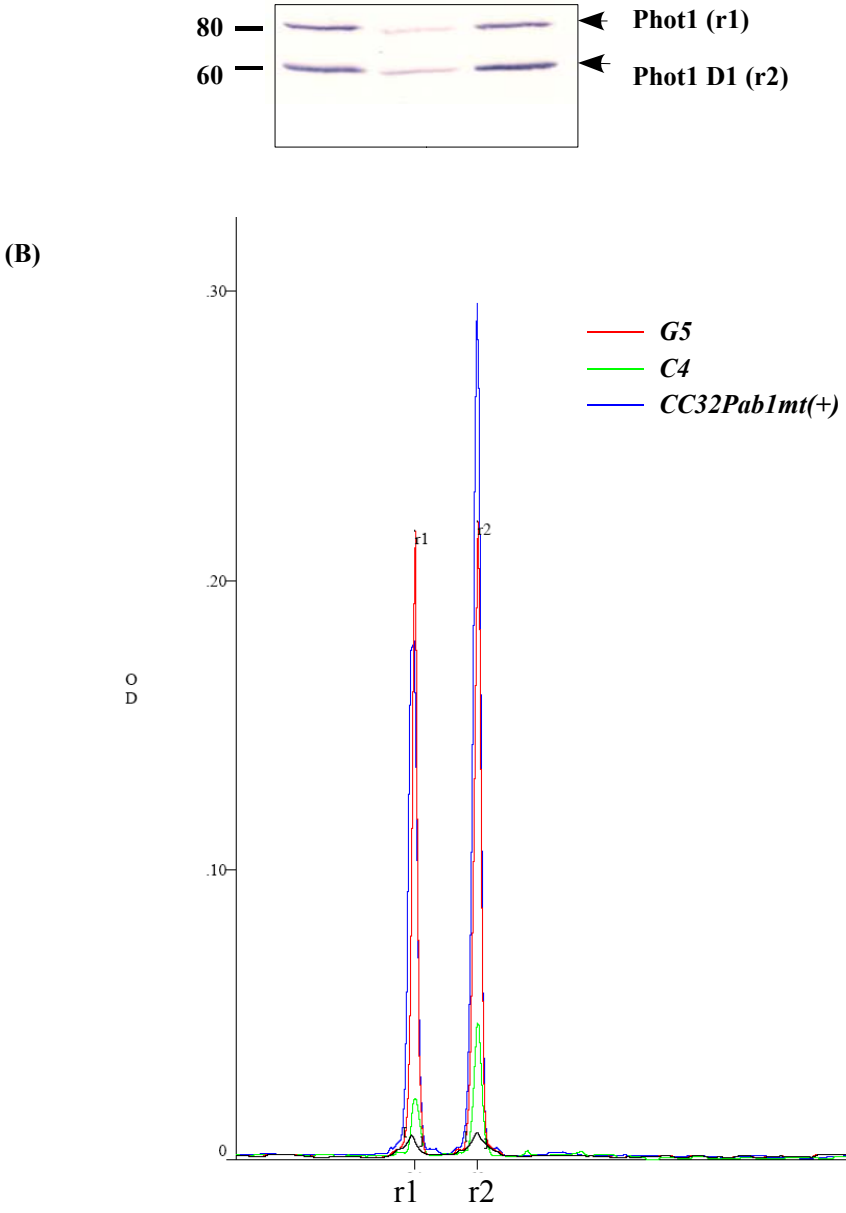


Figure 3.2.3.1 Relative phototropin amount in transformant, recipient and control strain.

(A) Western blot result of phototropin level in transformant, recipient and control strain. *C4* is the transformant with a pronounced reduction phototropin level. *G5* is the control strain in mating assay which only contain marker *aphVIII* gene. And *CC32pab1mt(+)* is the recipient strain. Same amount of protein (~80g) were loaded in each lane. An antibody raised against phototropin LOV1 domain was used. (B)Gel analysis result of the western blot. The blue line indicates the recipient strain *CC32pab1mt(+)*. The red line indicates the control strain *G5* and the green one indicates the transformant *C4*. The two peaks represent full length phototropin(r1) and its truncated version(r2). OD in the Y-axis is optical density. Both bands decreased in the transformants (*Gel-pro* analyzer was used for analysis.). Phot1, full-length phototropin (80kD); Phot1D1, degraded phototropin (60kD).

The transformant with pronounced reduction in phototropin level was named *C4*. Another transformant which only contained the marker gene *aphVIII*, was named *G5* and was used as a control strain in the mating assay

(Chapter 3.3). Full-length phototropin level of *C4* only counted for 10% of that in the recipient strain *CC32pab1mt(+)*and 10% of that in the control strain *G5*. The truncated phototropin in *C4* counts for 14% of that in the recipient strain and 19% of that in control strain *G5*. The total amount of Phototropin and its degraded version in *C4* counted for 13% of that in the recipient and 15% of that in the control strain *G5*. Both bands decreased in the phototropin RNAi construct transformant just as what happened in the transformants of *cw15 arg- A*, this phenomenon further confirmed the relationship of the two bands that both proteins might come from the same gene or there was very high homology between some part of the two protein. Since there is no second gene with high homology with phototropin was found in the *C.reinhardtii* Genome, the possibility increased that these two protein arised from the same gene.

3.3 Mating Assay

In *Chlamydomonas reinhardtii*, the importance of light in its sexual life has been known for a long time (Sager and Granick 1954; Martin and Goodenough 1975; Bernstein and Jahn 1955). Recently, Huang and Beck (2003), reported that phototropin was involved in gametogenesis, the maintenance of mating ability, and the germination of zygotes.

However, the traditional way about handling zygote germination is technically difficult and the relationship among light, phototropin and zygote germination need a better description. In this chapter, a new mating assay was set up to view the relationship from a different angle. A major advantage of this new assay is the easier handling and a better statistical evaluation.

3.3.1 The design of mating assay

The original strategy of this assay was to use two metabolic deficient strains as mating partners to complement each other. Those strains would not be able to grow on TAP medium without any additional nutrients. For zygote germination, there would be no background of unmated gametes so that the differences between Phot1- strain and the control strain can be easily compared.

Two pairs of mating partners (*CC48arg2mt(+)* and *CC645pab2mt(-), CC1930arg2 mt(-)* and *CC32pab1mt(+))* were chosen. *CC48arg2mt(+)* and *CC1930arg2 mt(-)* can not grow on TAP agar plates without addition of arginine. *CC645pab2mt(-)* and *CC32pab1mt(+)* can not grow on TAP agar plates without addition of p-aminobenzoic acid. Thus, unmated gametes can be easily distinguished. Because of the difficulty to obtain a RNAi construct transformants in *CC48arg2mt(+), CC645pab2mt(-)* or *cc1930arg2mt(-)* (Chapter 3.2), wild type strain *CC124mt(-)* was used as mating partner for *CC32pab1mt(+)* RNAi construct transformant *C4* and control strain *G5* (Chapter 3.2), both of which bear *aphVIII* genes. Those strains can not survive on TAP medium containing 15ug/ml paromomycin.

Gametes of both mating types are generated as described in the standard protocol. The gametes are then mixed and left to mate. Because of the metabolic deficiency of one parental strain and sensitivity to paromomycin of the other parental strain, neither of them can grow on the selecting plates. Thus the unmated gametes and vegetative cells are killed. Only the offspring of those germinated zygotes appear on plate. The plates are placed under continuous light for 3 to 4 weeks. The difference of Phot1- strain and control strain in zygote germination could be easily observed.

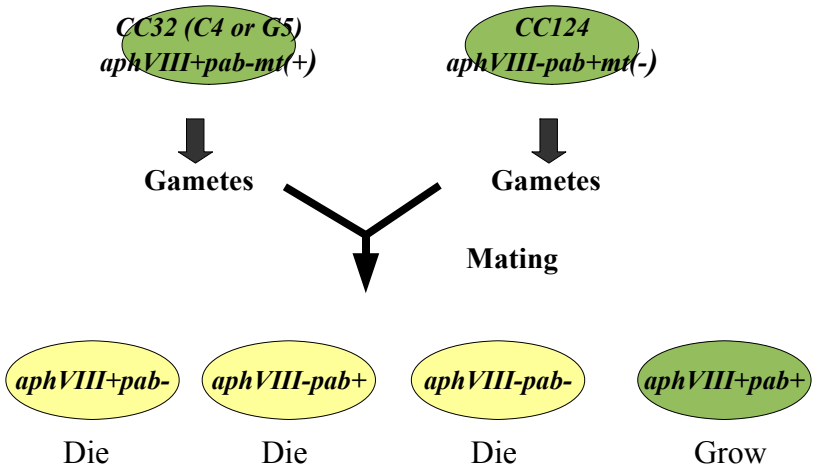
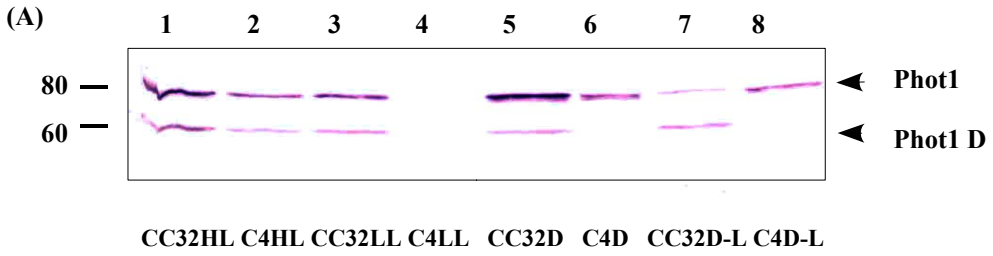


Figure 3.3.1.1 Description of mating assay

CC32pab1mt(+) transformants (*C4, G5*) and *CC124mt(-)* were chosen as mating partners. Gametes of both parts are prepared according to standard protocol. After mating, cell mixture are placed on minimal plates. Vegetative cells and unmated gametes will be killed. Zygote germination patterns will be compared between Phot1- strain and control strain.

3.3.2 Optimization of growth conditions for precultures before gametogenesis

RNA interference is different from knocking out the gene of interest by homologous recombination since the target mRNA level can not be completely eliminated. RNAi is a complicated process, different factors which influence the effect of RNAi keep changing during the cell cycle. Suppression of the target gene can not be fixed in a stable level. It became necessary to find out the best growing condition, under which the difference of the phototropin level is the biggest between *C4* and *CC32pab1mt(+)*.



(B)

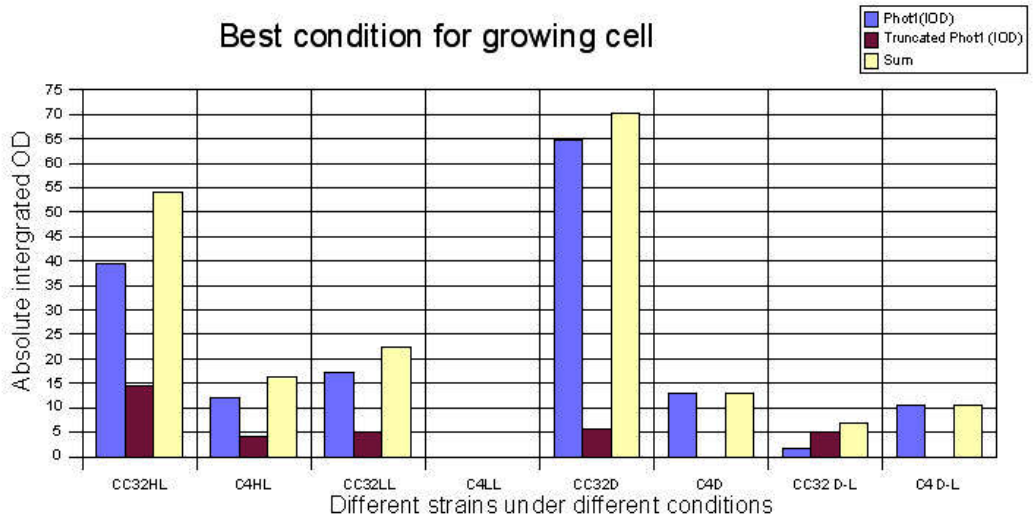


Figure 3.3.2.1 Difference in phototropin levels in transformant *C4* and recipient strain *CC32pab1mt(+)* under different growth conditions

(A) Western blot result of phototropin levels in *C4* and *CC32pab1mt(+)* grown under different conditions. Lane 1, 3, 5, 7: recipient strain *CC32pab1mt(+)*; lane 2, 4, 6, 8: transformant *C4.* Lane 1, 2: cells grown in high light conditions (~9W/m^2); lane 3, 4: cells grown in low light conditions (~1W/m^2); lane 5, 6: cells grown in complete darkness; lane 7, 8 : cells first grown in darkness for two days and then subsequently in middle light condition (~4W/m^2). Same amount of protein (100μg) of cell lysate was loaded in each lane. Antibody raised against phototropin LOV1-domain was used. Phot1, full-length phototropin (80kD); Phot1D, degraded phototropin (60kD). (B) Analysis of the western blot result. HL: high light conditions; LL: low light conditions; D: darkness; D-L: incubation in middle light conditions after transfer from darkness. From the western blot result, it was clear that phototropin levels in the transformant and the recipient strain varied as the growth conditions changed. Although under high light condition, low light condition and complete darkness, the levels of phototropin in the transformant were always lower than those in the recipient strain, under low light condition, phototropin level in the transformant was the lowest. Thus, low light condition (~1W/m^2) was chosen to grow vegetative cells before mating. IOD indicates integrated optical density. *Gel-pro* analyzer was used for analysis.

Four different conditions were tested: high light (~9W/m^2), low light (~1W/m^2), darkness, darkness for 2 days and then subsequently in middle light (~ 4W/m^2) for another 2 days. All the cells were grown at 25ºC with continuous shaking (120rpm).

Cells were collected at late log phase. Equal amount of protein (100μg per lane) from whole cell lysate was loaded on 12% SDS gel, and an antibody raised against phototropin LOV1-domain was applied to test the level of Phot1. It was found that under high light conditions, low light conditions and complete darkness, transformant *C4* always had a lower phototropin level, which proved that the RNAi construct could reduce phototropin level under those conditions.

But the efficiency seemed to be different in those three situations. Grown under high light, full length phototropin level in *C4* was 30% of that in recipient strain, and truncated Phot1 level in *C4* was 27% of that in

recipient strain. Grown under low light condition, Phot1 and its degraded product both reduced to almost zero in the transformant. Grown in darkness, the band of truncated Phot1 in *C4* was almost invisible while the full length phototropin in *C4* still counts for 20% of that from the recipient strain. When *C4* and *CC32pab1mt(+)* were first grown in darkness and then transferred to middle light condition, phototropin in *C4* is even more abundant than in *CC32pab1mt(+)*.

The different efficiency of RNAi in transfomant *C4* under different conditions suggested the necessity of selecting the growth conditions. From the result described above, the low light condition (~1W/m^2) was chosen for growing preculture and for gametogenesis since the phototropin level (both full length phototropin and truncated phototropin) was close to zero.

3.3.3 Mating assay results

Zygote germination is a complicated process, in which both phototropin and light are the key players. Phototropin is also involved in gametogenesis and maintaining mating compentence (Huang et al., 2003). To prove the involvement of phototropin, it is important to get rid of the impact caused by difference in gametogenesis efficiency of Phot1- strain and control strain. More importantly, the steps of manipulation should be reduced to avoid man-made mistakes during experiments. The mating assay used in this study fulfilled these two requirements.

mt(+) gametes were made from *C4 (Phot1-pab-aphVIII+)* and *G5 (Phot+pab-aphVIII+)*, while mt(-) gametes were made form wild type strain *CC124 mt(-) (Phot1+pab+aphVIII-)*. One hour was given to let motile and competent gametes form pairs and fuse. After that, mating mixtures were plated on selective plates to stop pairing and separate the already formed zygotes. The plates were then placed under different light intensities for few weeks. Unmated gametes and vegetatively grown cells would be killed either by paromomycin or lack of p-aminobenzoic acid.

All the *C4* offspring were regarded as Phot1- zygotes while all the *G5* offspring were regarded as Phot1+ zygotes.. The reason lies in the mechanism of RNA interference. When hairpin dsRNA is degraded into siRNA, RdRp could use the single-stranded siRNAs as primers and use phototropin mRNAs as templates to synthesize double-stranded RNA and enter the cycle again.

Three different light intensities were used for zygotes germination: HL (high light condition: ~9W/m^2), ML (middle light condition: ~4W/m^2) and LL (low light condition: ~1W/m^2). The plates are placed under these conditions continuously for several weeks. The result obtained is shown in Figure 3.3.3.1.

It was discovered that both light intensity and phototropin level are relevant to the germination of zygotes. Among them, the light intensity applied to zygotes was the main factor. Under high light condition and low light condition, zygotes germination results showed almost no difference between *C4* plus *CC124mt(-)* and *G5* plus *CC124mt(-).* Under low light conditions, very low zygote germination rate suggested that the applied light intensity was the key factor. Under high light conditions, offspring from both *C4* and *G5* had very similar zygote germination pattern although the phototropin levels were different, which suggested that the applied light intensity saturated at both phototropin levels. Only under middle light condition, *C4* offspring germinated more slowly than *G5* offspring, which proved the involvement of phototropin in zygote germination.

As time passed, the nutrient substance on those plates became depleted and the cells started to die, which is the reason causing a climax of cell number. Under high light conditions, the zygote germination and cell bleaching of *C4* and *G5* offspring seemed to by 'synchronized'. Under low light conditions, the germination rate was low and cell growth were very slow, the nutrients depletion was also slow. No obvious difference

was observed between offspring of *C4* and *G5*. While under middle light condition, there seemed to be a phase shift between *C4* and *G5* offspring if the time of cell number climax was used as a standard. The cell number climax of *C4* offspring appeared slower than that of *G5* offspring. The phase shift between C4 offspring and G5 offspring could be explained only by the difference of phototropin level.

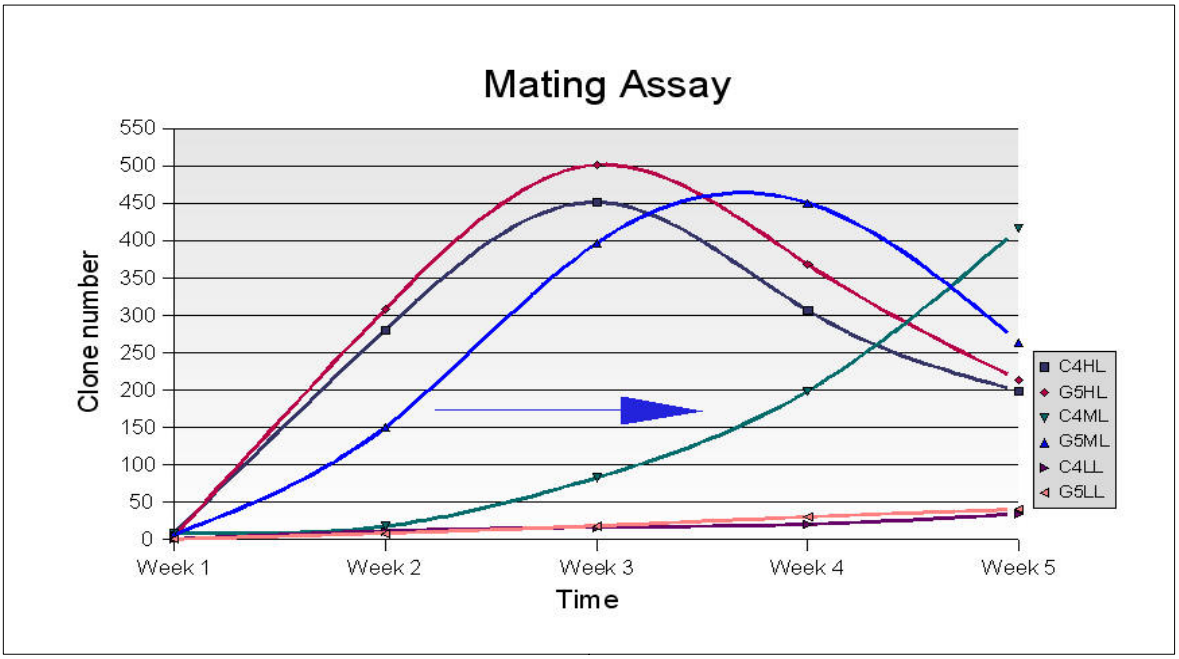


Figure 3.3.3.1 Result of mating assay

The Y axis indicates the clone number per plate and the X axis indicates the time. Different colors represent different strain offspring and different growth conditions. Under high light conditions, very little difference appeared between the offspring of transformant *C4* and control strain *G5*. Under middle light condition, the zygote germination of *C4* offspring seemed to be slower than that of *G5* offspring. Under low light, offspring of both strains had very low zygote germination rate.

By this mating assay, the involvement of light and phototropin in zygote germination was further proved and studied from another viewpoint. Light intensity is the key factor for zygote germination. Low light intensity would hardly trigger zygote germination in both kinds of cells, i.e. cells with high or low phot1 levels. Under moderate light intensity, phototropin level becomes the key factor for the germination process. Those zygotes which have more phototropin and sense more light would germinate earlier.

3.4 Expression and purification of phototropin in different expression systems.

3.4.1 Expression of Phototropin in Oocytes

Xenopus laevis, the South African clawed frog, is a popular model organism. Its oocytes are able to translate efficiently and faithfully foreign genetic information and capable of assembling oligomeric receptor/channel complexes and inserting them into the plasma membrane (Lester, 1988). The oocyte system was chosen for expressing phototropin for two reasons. First, it was very difficult to overexpress phototropin directly in *C. reinhardtii*. Different constructs for overexpressing phototropin directly in *C. reinhardtii* and different *C. reinhardtii* strains had been tested and intensive screenings were applied as well to more than one thousand colonies. Not a single clone with greatly improved phototropin level was detected (data not shown). Second, although light-induced degradation experiment and RNAi construct transformant have shown the close relationship between full length phototropin (80kD) and the suspected degraded phototropin (60kD), direct proof was still in need to show that the two protein originated from the same gene.

In almost all LOV domains, the amino acid sequence NCFLRQ (Asn-Cys-Arg-Phe-Leu-Gln) is highly conserved. Residue Cys is in charge of forming an adduct with FMN by a covalent bond when phototropin gets activated. When serine was used in place of cysteine, LOV domain remained in its inactive form (Swartz et al., 2001). The light induced degradation experiment (Chapter 3.1) showed that the 60kD band could be upregulated by strong illumination. Therefore it was postulated that before activation by light, the inactivated LOV1 and LOV2 domains cover the potential protease site of the kinase domain. Therefore, phototropin was resistant to protease. When activated by blue light, the conformational change of the LOV1 and LOV2 domain make protease site to be exposed so that the C-terminus of phototropin could be degraded. Two different phototropin expression constructs were made. One was for expressing wild type phototropin and the other contained a phototropin mutant with two Cys changed into Ser (C57S, C250S). It was expected that the second band would not appear in the oocyte which expressed phototropin (C57S, C250S), if the degradation was caused by light induced conformation change.

The Cys to Ser mutation was introduced into both LOV domains of *C. reinhardtii* Phototropin by PCR. Both *phototropin* cDNAs and *phototropin* (C57S, C250S) mutant cDNAs were cloned into the vector pGEM-RE, which is a modified pGEM-HE (Liman et al., 1992) with extended cloning sites. The inserts were placed between the BamHI and EcoRI restriction sites, and on the 5' ends, DNA sequences encoding 8 His-tag were introduced.

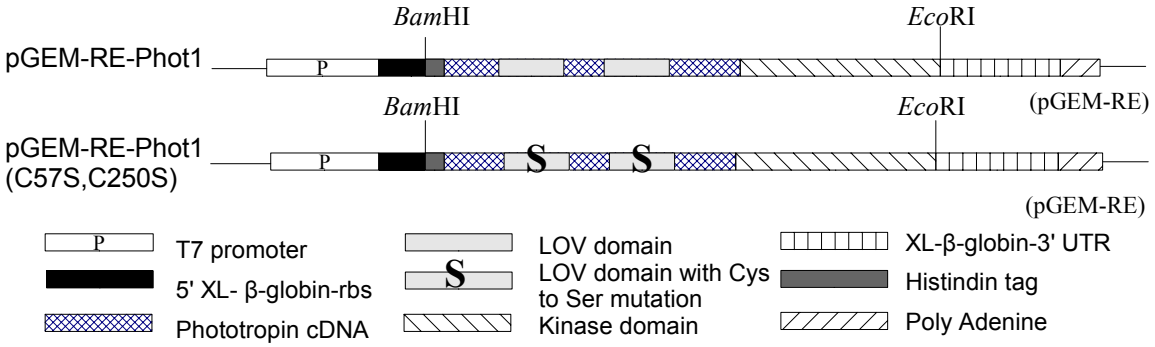


Figure 3.4.1.1 Schematic drawing of the two plasmids used for expression of Phot1 and Phot1 (C57S, C250S) in *Xenopus laevis* oocytes. Different color boxes represent different fragments. Restriction enzymes are indicated above the construct. The plasmid vector is indicated on the right side of the vector.

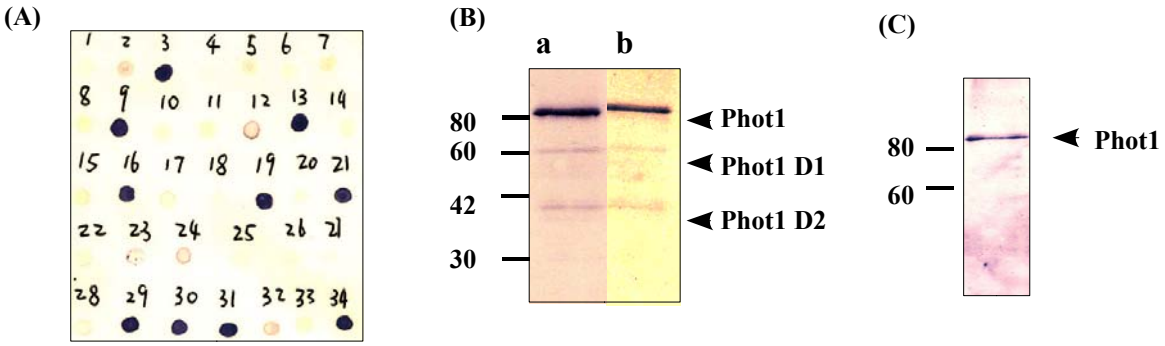


Figure 3.4.1.2 Expression of Phot1 and Phot1 mutant (C57S, C250S) in *Xenopus Laevis* oocytes.

(A) Dot blot result. Different oocyte lysates were dropped on the membrane in equal amount. Antibody against phototropin LOV1 domain was used for detecction. Although injected with same amount of mRNA, different oocyte had different expression level. (B) The degradation pattern of phototropin and its mutant. An antibody against phototropin LOV1-domain was used for detection. Both phototropins could be degraded, and two C-terminus degraded products were detected. One was around 60kD and the other was around 35kD. Lane a: wild type phototropin expressed by oocyte. Lane b: phototropin mutant expressed by oocyte. (C) Western blot result of oocyte expressed wild type phototropin. Anti-His tag monoclonal antibody was used to detect the His tagged expression product. Phot1, full-length phototropin (~80kD); Phot1 D1, degraded phototropin (~60kD); Phot1 D2, degraded phototropin (~35kD).

Dot blot was used to find out those oocytes which expressed the target protein (Figure 3.4.1.2 A). It was found that not all the injected oocytes expressed Phot1 at the same level and some even did not express phototropin at all. An anti-His tag monoclonal antibody was used to confirm the expression of phototropin (Figure 3.4.1.2 C).

In those oocytes which expressed target protein (Figure 3.4.1.2 Ba), the 35kD and 60kD degradation products could be detected with antibody against phototropin LOV1-domain. The 35kD band had been detected before only in *C. reinhardtii* strain cw15 arg- A (Chapter 3.1, Figure 3.1.1.2 B). The result confirmed the postulation that the full length phototropin (~80kD) and the lower band (~60kD) originated from the same gene. The N-termini of the proteins could be recognized the same antibody. The 60kD degradation product (~60kD) could be further degraded at its C-terminus into a 35kD product.

In those oocytes which expressed Phot1 (C57S, C250S), the degradation products could also be detected (Figure 3.4.1.2 Bb). Since the LOV domains in this mutant can never be activated, this result suggested that the degradation did not require the activation of the LOV domains. The further degradation of the truncated phototropin was also independent of the activation of LOV domains.

In oocytes, some portion of the expressed Phot1 existed as soluble protein. Still, the major part of the expression product attached to the oocyte membrane. Very small amount of membrane attached Phot1 could be solubilized by non-ionic detergent NP-40 (Figure 3.4.1.3 A). The C-terminal truncated phototropin was

easy to get degraded in oocyte even in the presence of protease inhibitors. Those oocytes which expressed Phot1 (C57S, C250S) were not tested due to their low expression levels.

In *C. reinhardtii*, phototropin was down regulated by degradation when strong illumination was applied to the cells (Chapter 3.1, Figure 3.1.3.1). Similar degradation experiment was carried out to test whether in oocyte the degradation of Phot1 could be induced by illumination. (Figure 3.4.1.3 B). Due to the difference in expression levels in different oocytes, oocytes which had been injected with Phot1 cRNA were first screened by dot blot. Those oocytes which strongly expressed Phot1 were collected and thoroughly resuspended. The mixture was divided into 5 aliquots, one was frozen in -80ºC as control (C) and the others were kept in room temperature under different light conditions. One sample (L1) was exposed to strong light (~10W/m^2) for one day and then frozen at -80ºC and a control sample (D1) was kept at the same temperature but covered with aluminum foil. Another sample (L4) was exposed to strong light for four days and a control sample (D4) covered with aluminum foil was placed next to it. On the western blot (Figure 3.4.1.3 B), phototropin in L4 totally disappeared after four days of strong illumination, while phototropin levels in C, D1, D4 and L1 did not differ much. Same as in Chlamydomonas, obvious degradation happened at least after 24 hours. The degraded phototropin was missing in all the samples, which could be caused by its instability. Due to the poor expression of phototropin (C57S, C250S), this experiment wasn't carried out with the mutant.

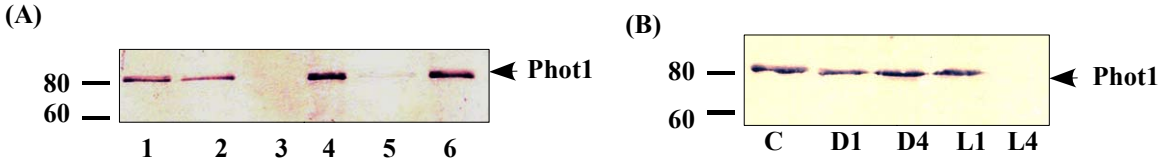


Figure 3.4.1.3 Distribution and solubilization of Phot1 expressed in oocyte and light induced degradation experiment.

(A)Western blot of distribution and solubilization of phot1 expressed in oocytes. Lane 1: whole cell lysate of oocytes, Lane 2: soluble fraction of oocytes, Lane 3: Protein washed off from oocytes membrane by HbA buffer containing 1% n-Octyl-β-D-glucopyranoside, Lane 4: Oocyte membrane fraction left after the wash with HbA buffer containing 1% n-Octyl-β-D-glucopyranoside, Lane 5: Protein washed off from oocytes membrane by HbA buffer containing 1% NP-40, Lane6: Oocyte membrane fraction left after the wash with HbA buffer containing 1%NP-40. Same amount of protein was loaded in each lane. Antibody against phototropin LOV1-domain was used.(B)Western blot of light induced degradation experiment of phot1 in oocytes. Lane M: Marker, Lane C: Oocytes lysate control which was kept at -80C, Lane D1: Oocytes lysate which was kept in darkness at room temperature for one day and then transferred to -80C, Lane D4: Oocytes lysate which was kept in darkness at room temperature for 4 days and then transferred to -80C, Lane L1, Oocytes lysate which was kept in strong light at room temperature for 1 day and then transferred to -80C, Lane L4: Oocytes lysate which was kept in strong light at room temperature for 4 days and then transferred to -80C. Same amount of oocytes lysate was applied in each lane. The light intensity was ~10W/m^2. Antibody against phototropin LOV1-domain was used. Phot1, full-length phototropin.

In conclusion, the expression of Phototropin in oocytes confirmed the hypothesis that both bands (80kD and 60kD) detected in western blot of *C.reinhardtii* lysate were products of one gene. Phototropin was also partially soluble in oocytes and the membrane attached phototropin could be solubilized by nonionic detergent NP-40. Phototropin could exist in two conformations, of which one is resistant to protease and the other is sensitive to protease. Both conformations exist in parallel, which is the reason that both Phot1 and Phot1 (C57S, C250S) had a C-terminus degradation. Strong illumination increased the formation of phototropin which was sensitive to protease. The degraded phototropin was unstable and did not accumulate

as the full length phototropin got degraded.

3.4.2 Fusion expression of Ble-Phot1 in *C. reinhardtii*

Till now, full-length phototropin has not been reported to be purified successfully from any plant cells. Although it is possible to use insect cells to express phototropin, the high cost for production limited the in vitro studies of phototropin. As a promising expression system, *C. reinhardtii* cells were chosen to express Phot1.

Several different constructs were made to express phototropin fused with Ble gene (Streptoalloteichus hindustanus Bleomycin resistance protein) or to express phototropin directly in *C. reinhardtii.*

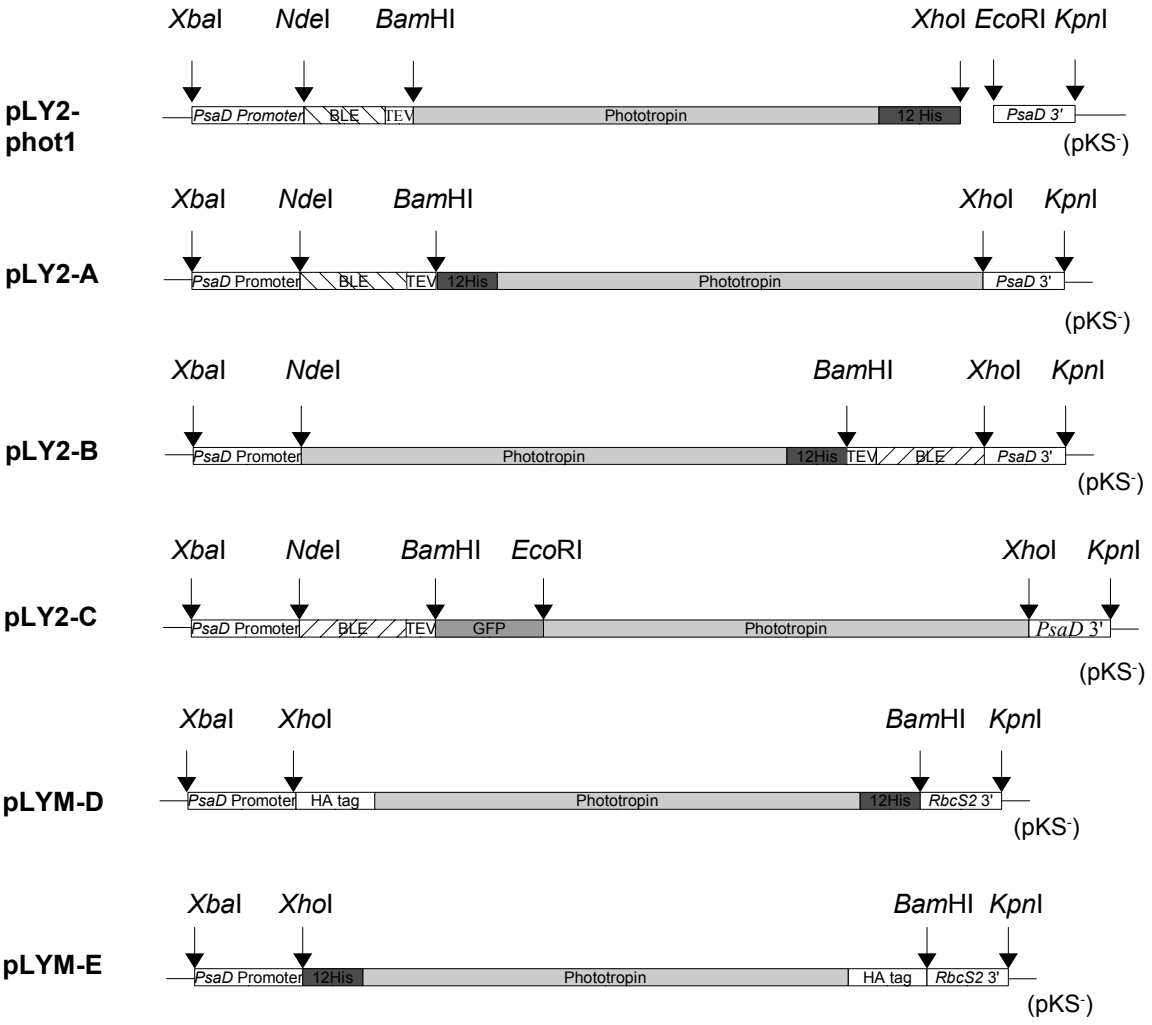


Figure 3.4.2.1 Different constructs tested for expressing Phototropin in *C. reinhardtii*

Six different constructs were made as shown above. Different color boxes represent different fragments. The restriction enzymes used for cloning are indicated above each construct. The plasmid vector is indicated on the right side of each construct. BLE, zeocin binding protein; GFP, green fluorescence protein; TEV, Tobacco Etch Virus protease site; 12His, Histidine tag; PsaD 3', PsaD 3'UTR; RbcS2 3', RbcS2 3' UTR.

In the construct pLY2-phot1, phototropin was fused with the marker gene Ble so that transformants could be

directly screened on plate. Ble was in the N-terminus of the fusion product and a TEV protease site (Glu-Asn-Leu-Tyr-Phe-Gln↓Gly) was placed between the two proteins so that further cleavage could separate the two parts. A 12-His tag was placed in the C-terminus of the fusion product for future purification. In order to construct such an expression vector, a 987bp BamHI-XbaI fragment containing PsaD 5' upstream region and Ble-TEV was subcloned into pKS-. And a BamHI-EcoRI fragment containing Phot1 cDNA and a 12-histidine-tag was subcloned behind it. The PsaD 3'-downstream region was placed in the end between XhoI and KpnI. pLY2-A still used almost the same strategy as in pLY2-phot1. The difference was that a 12His tag was inserted between the protease TEV site and phototropin N-terminus. In pLY2-B, Ble gene was placed in the C-terminus of the fusion product. Thus the full length phototropin was expected to be expressed. In pLY2-C, a green fluorescence protein (GFP) was placed between TEV site and phototropin N-terminus. It was made to find out the localization of the expressed product. pLYM-D and pLYM-E were constructs for testing the possibility of expressing Phot1 alone. HA tag (YPYDVPDYA) and 12 His tag were placed on either side of phototropin for detection and purification in case of C-terminal degradation.

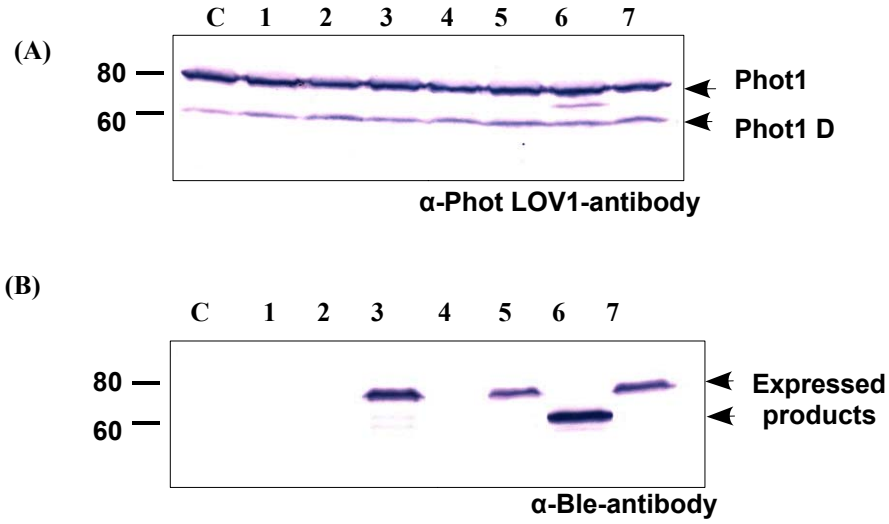


Figure 3.4.2.2 Overexpression of fusion protein Ble-TEV-Phot1-12 His in *C. reinhardtii*

(A) Western blot of screening by using antibody against phototropin LOV1 domain. (B) Western blot of same sample detected by antibody against Sh-Ble. Lane C, recipient strain, Lane 1-7: different transformants obtained. Equal amount of whole cell lysate(~100µg) was loaded each lane. Only transformant 6 could be detected by both antibodies. Transformants 3,5 and 7 could only be detected by anti-Sh-Ble antibody. Phot1, full-length phototropin (~80kD); Phot1 D, degraded phototropin (~60kD).

pLY2-phot1, pLY2-A, pLY2-B and pLY2-C were transformed into *C. reinhardtii* strain cw15 arg- A. pLYM-D and pLYM-E were co-transformed with pSI103 into cw15 arg- A.

Several transformants of pLY2-phot1 were obtained (Figure 3.4.2.2). Only one transformant, No. 6, had the expression product that could be recognized by both the antibody against *Sh* Ble and the antibody against Phot1 LOV1-domain. Unfortunately, the expressed product (~70kD) had an obviously smaller molecular weight than prediction (94kD), which suggests that the product is not a full-length product. Transformant No.3, 5 and 7 could only be recognized by the anti-*Sh* Ble antibody and the product was around 80kD, which is smaller than estimated molecular weight (94kD). Unlike what happened with oocyte expressed phototropin or *Chlamydomonas* endogenous phototropin, the expected C-terminal degradation product (~60kD) did not

appear in any of the transformants. The possible explanation could be that the degraded fusion expression product was very easy to be destroyed. The result obtained in this experiment shed light on the possibility of expressing phototropin in *C. reinhardtii*.

From the colonies obtained from pLY2-B and pLY2-C, several clones which contained the expressed product with right size and degradation pattern were detected by an antibody raised against Sh-Ble. But further tests with the antibody against phototropin LOV1-domain showed that they were not the right transformants. No transformants of pLYM-D and pLYM-E were obtained although intensive screening had been performed. Only colonies obtained from pLY2-A transformation had the expressed product with the right size and degradation pattern as predicted. Both the antibody against *Sh* Ble and the antibody against phototropin LOV1-domain could recognize the expressed product. Out of 80 colonies obtained, 66 had the correct product.

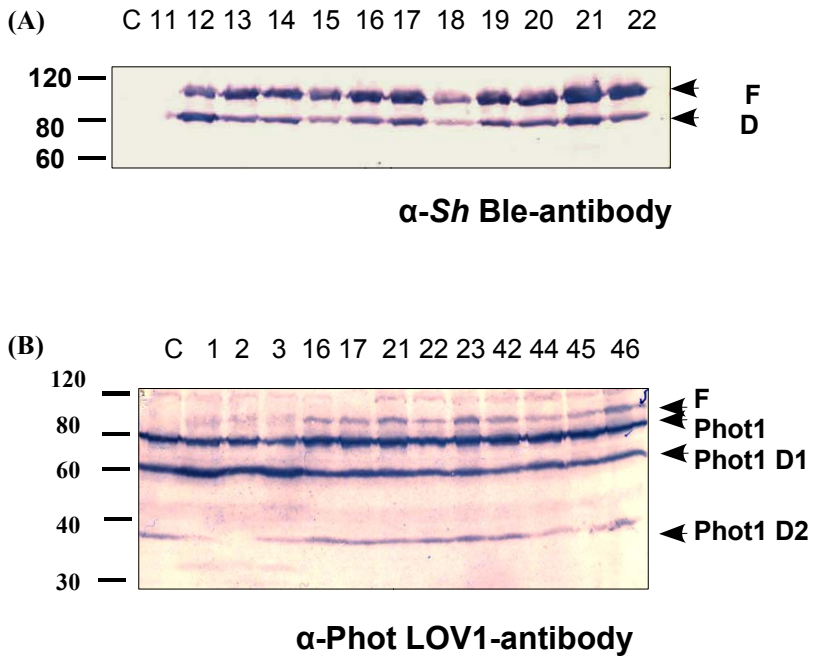


Figure 3.4.2.3 Western blot of pLY2-A transformants.

(A) Western bolt screen results detected with antibody against *Sh*-Ble. Out of 80 transformants, 66 clones have the expressed product with the right size as predicted and similar degradation pattern as phototropin. Screen result of transformant no.11-22 was shown. Lane C, whole cell lysate of recipient strain *cw15 arg- A*; Lane 11-22: whole cell lysate of transformants 11-22. Same amount of cell lysate was loaded in lane C and lane 11-22. (B) Western blot of selected transformants detected by antibody against *C. reinhardtii* phototropin LOV1 domain. Transformants no. 1, 2, 3, 16, 17, 21, 22, 23, 42, 44, 45, 46 were selected for the test. In almost all selected transformants, the full length fusion expression product could be detected. Same amount of cell lysate was loaded in lane C (recipient strain *cw15 arg- A*) and lane1, 2, 3, 16, 17, 21, 22, 23, 42, 44, 45, 46. The truncated over-expressed product has the same molecular weight as phototropin and could hardly be distinguished from the band of full-length phototropin. F, full-length expression product (~94kD); D, degraded expression product (~74kD); Phot, full-length phototropin (~80kD); Phot1 D, degraded phototropin (~60kD); Phot1 D1, degraded phototropin (~35kD).

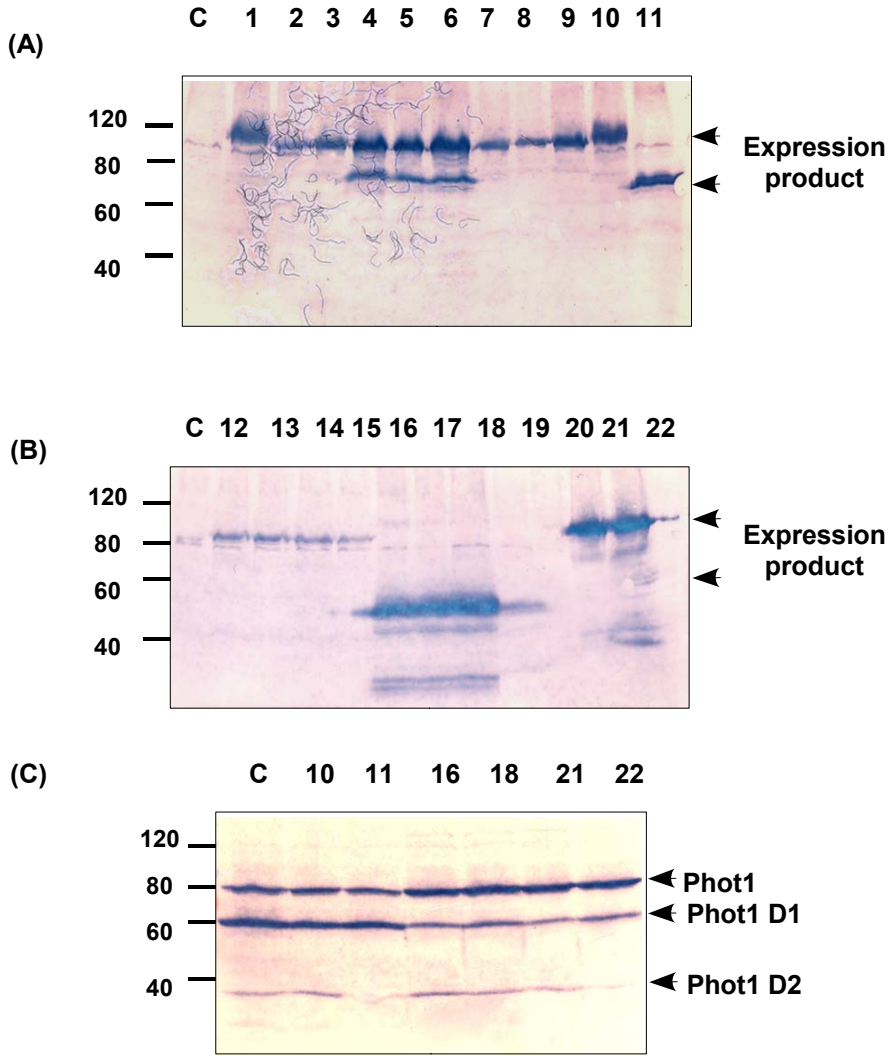


Figure 3.4.2.4 Western blot result from screening of pLY2-B transformants

(A), (B): Western blot results detected by an antibody raised against *Sh*-Ble. Several colonies with pronounced expression were found. (C): Western blot results of selected colonies detected by antibody raised against phototropin LOV1-domain. No transformant was detected. Lane C, strain *cw15 arg- A* lysate; Lane 1-22, different transformant lysate. Equal amount of cell was loaded in lane C and lane 1 -22. Although the anbibody against *Sh*-Ble could detect some fusion expression product, the phototropin part was missing in them. Those product could caused by radom integration during transformation. Phot1, full-length phototropin (~80kD); Phot1 D, degraded phototropin (~60kD); Phot1 D1, degraded phototropin (~35kD).

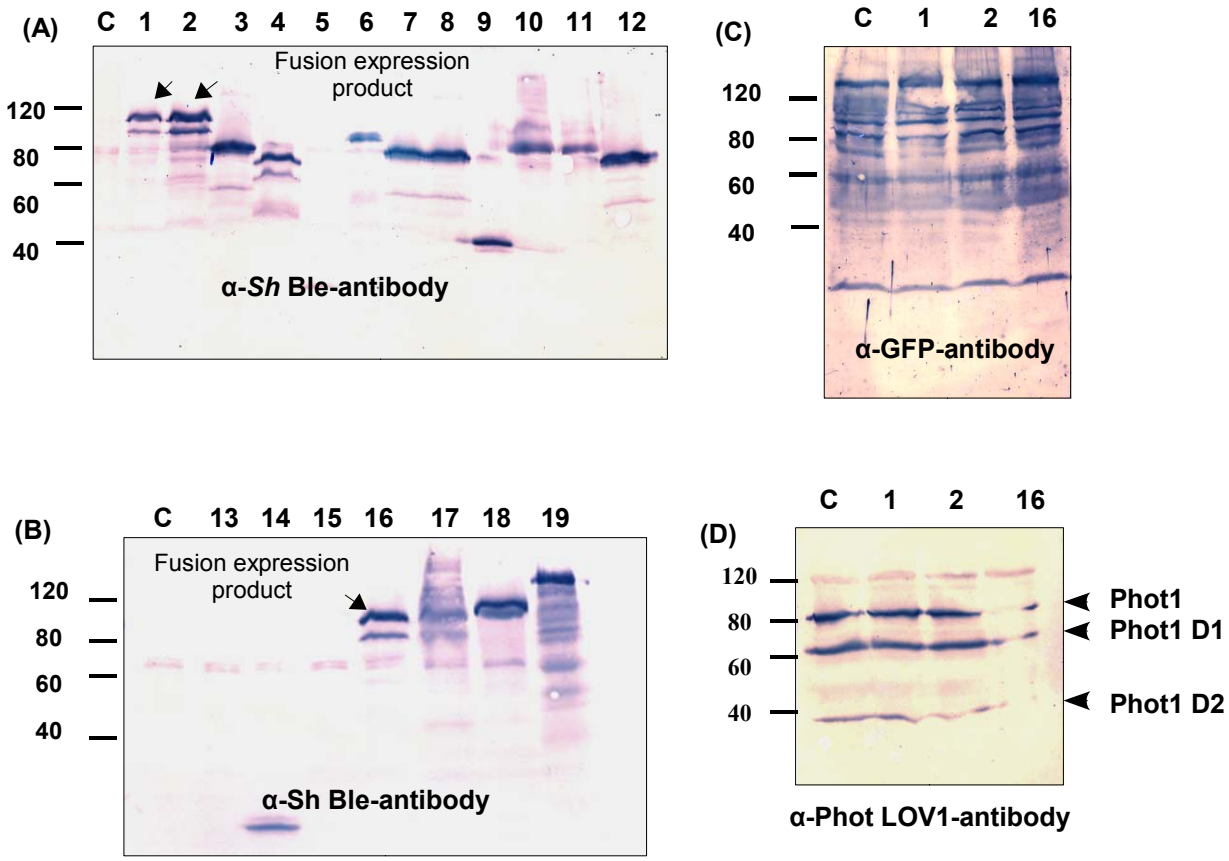


Figure 3.4.2.5 Western blot result from screening of pLY2-C transformants

(A), (B): Western blot of screening results detected with the antibody against *Sh* Ble. Only a few colonies (1, 2, 16) had fusion expression products with correct molecular weight (115kD) and estimated degradation pattern. (C): Western blot results of selected colonies 1, 2, 16 detected by antibody against GFP. No fusion expression products could be detected. (D): Western blot of transformants 1, 2, 16 detected by antibody against phototropin LOV1-domain. No fusion expression product was detected. Lane C, whole cell lysate of recipient strain *cw15 arg- A* ; Lane 1-19, whole cell lysate of different colonies. Equal amount of protein (~100µg) was loaded in lane C and lane 1-19. Phot1, full-length phototropin (~80kD); Phot1 D, degraded phototropin (~60kD); Phot1 D1, degraded phototropin (~35kD).

3.4.3 Analysis of pLY2-A transformant No.21

The fusion expression product had the correct molecular weight and degradation pattern. Still, it was important to prove the identity of the expressed product. For this purpose, the transformant 21 (Figure 3.4.2.3) was picked to perform a series of tests.

3.4.3.1 Distribution of the expressed product and the endogenous phototropin

The transformant was grown under the same condition as the recipient strain *cw15 arg- A* until the stationary stage. After cell harvest, the transformant 21 and recipient strain were fractionated as described in Huang et al. (2002) into low speed pellet fraction (P), membrane fraction (M) and soluble fraction (S). Equal amounts

of protein from different fractions were loaded on SDS-PAGE for western blot. Antibodies raised against both *Sh* Ble and *C. reinhardtii* phototropin LOV1-domain were used for detection. It was found that both endogenous phototropin and expressed product were present in all the three fractions.

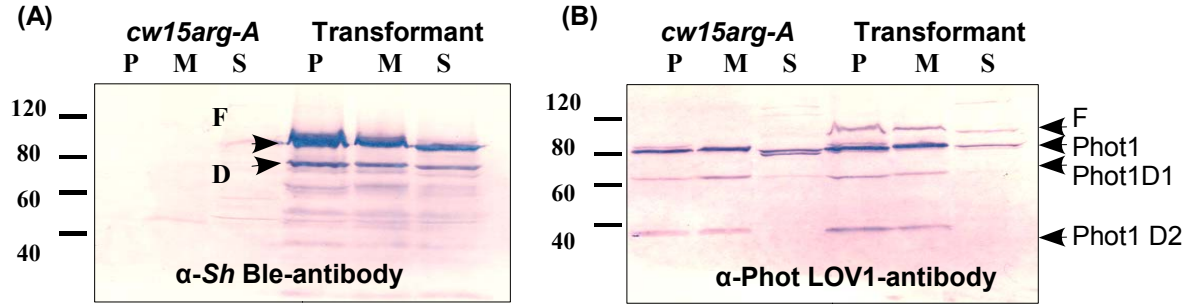


Figure 3.4.3.1.1 Distribution of fusion expression product and phototropin

(A) Western blot result of distribution of fusion product in pellet fraction (P), membrane fraction (M) and soluble fraction (S) detected by an antibody against *Sh* Ble in *cw15arg-A* and the transformant 21. Both the full length (94kD) and the truncated fusion products (74kD) were present in membrane attached form and soluble form. (B) Western blot result of distribution of phototropin and the fusion expression product in pellet fraction (P), membrane fraction (M) and soluble fraction (S) detected by the antibody against phototropin LOV1-domain in recipient strain and the transformant. Lane P: pellet fraction; Lane M: membrane fraction; Lane S: soluble fraction. Equal amount of protein (100μg) was loaded in each lane. F: full length fusion expression product (~94kD); D: degraded fusion product (~74kD); Phot1, full-length phototropin (~80kD); Phot1 D1, degraded phototropin (~60kD); Phot1 D2, degraded phototropin (~35kD).

Form 3.4.3.1.1 Distribution of phototropin and the fusion expression product.

	Distribution in Pellet fraction, Membrane fraction, Soluble fraction
Phototropin in recipient strain (detected by antibody against Phot LOV1)	*1.9 : 1.9 : 1*
Fusion expression product in transformant (detected by antibody against *Sh* Ble)	*2.1 : 1.4 : 1*
Fusion expression product in transformant (detected by antibody against Phot LOV1)	*3.8 : 2.5 : 1*

With the assistance of the *Gel-pro analyzer*, the distribution of phototropin and expressed product was determined. It was found that the distribution of fusion expression product was very close to that of phototropin. Although the distribution of fusion expression obtained by different antibodies varied, it was obvious that the anti *Sh* Ble antibody was more sensitive than the anti Phot LOV1-domain antibody in recognizing fusion product.

Same as phototropin, the fusion product in the soluble fraction had a smaller molecular weight than those in the pellet fraction and membrane fraction (~3kD). The difference was more obvious than that between soluble phototropin and membrane-attached phototropin. The fusion expression product also had a C-terminus degradation pattern. The difference between full length product (94kD) and degraded product

(74kD) was close to that between full length phototropin (80kD) and degraded phototropin (60kD).

Different from the degradation product of phototropin, the degraded fusion product (74kD) also appeared in the soluble fraction of the transformant (Figure 3.4.3.1.1 A). Its molecular weight was smaller than its counterpart in low speed pellet fraction and membrane fraction (~3kD). The difference could be caused by modification.

3.4.3.2 Light induced degradation of the fusion protein

As described in Chapter 3.1, phototropin exhibited a light-induced down regulation. Under strong light, full length phototropin became degraded while the 60kD product accumulated; while in low light condition or darkness, the full length phototropin counted for the majority of the two kinds of phototropin. The down-regulation process took more than 48 hours. A light-induced degradation experiment was carried out with the transformant 21 (Figure 3.4.3.2.1 A).

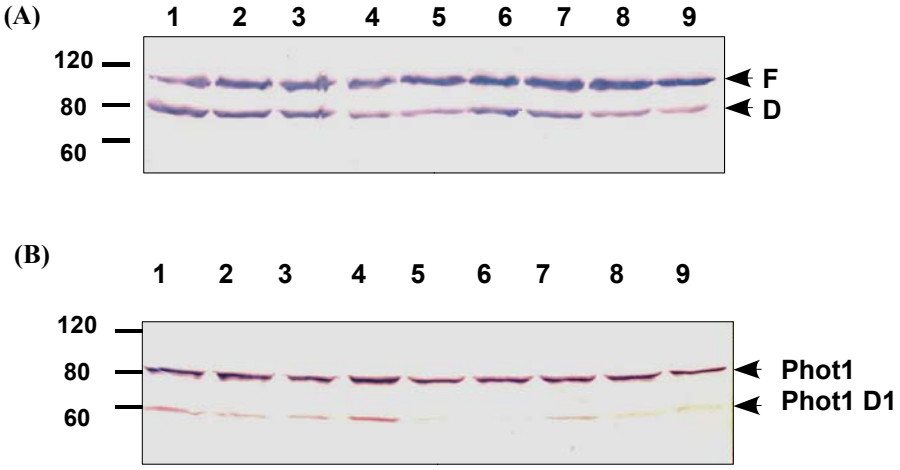


Figure 3.4.3.2.1 Light gradient test for overexpression product and phototropin

(A) Western blot detected by anti *Sh* Ble antibody. Sample 1-9 were all pLY2-A transformant 21, which were exposed to a light gradient (17600mW/m^2, 10000mW/m^2, 6300mW/m^2, 3300mW/m^2, 1800mW/m^2, 870mW/m^2, 510mW/m^2, 260mW/m^2, 90mW/m^2) continously for 3 days. Same amount of cells were loaded in each lane. (B) Western blot detected with antibody against phototropin LOV1-domain. The same samples as in (A) were used. Same amount of cells were loaded in each lane. F, full-length fusion product (~94kD); D, degraded fusion product (~74kD); Phot1, full-length phototropin (~80kD); Phot1 D, degraded phototropin (~60kD).

Similar degradation pattern under different light conditions also appeared in the fusion expression product. More degraded expression product (74kD) was detected under strong light while under low light, full length fusion product (94kD) counted for the majority. This suggested the fusion protein was regulated in the similar

way as the endogenous phototropin.

3.4.3.3 Purification of the fusion protein

To prove the identity of the fusion product, further experiments were required. Since there was a 12-histindine-tag between Ble and Phototropin, Ni-NTA resin could be used to purify the fusion product. To increase the accessibility of Ni^{2+} to histindine, denaturing purification conditions were used. Unlike those recombinant proteins with His tag in N-terminus or C-terminus, the fusion product with His tag in the middle was difficult to purify even under denaturing conditions.

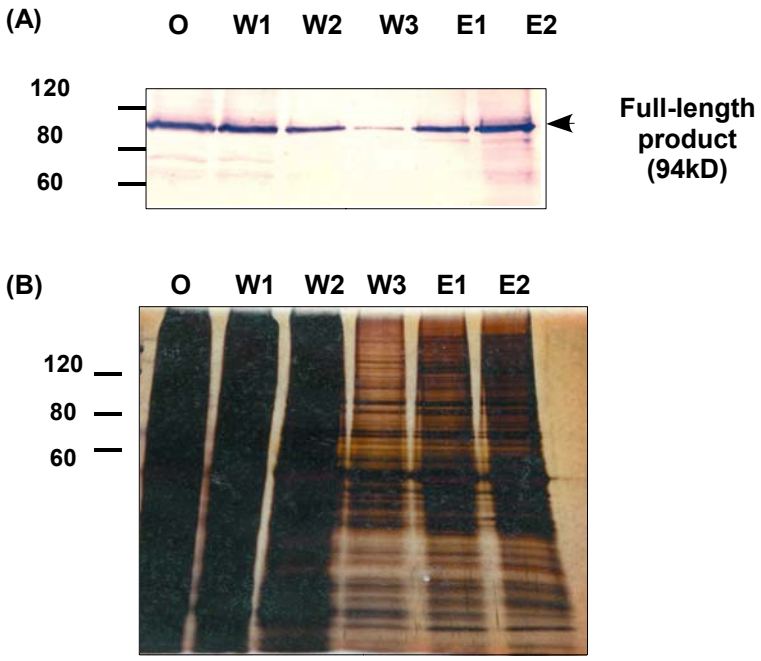


Figure 3.4.3.3.1 Ni-NTA spin column purification of fusion product.

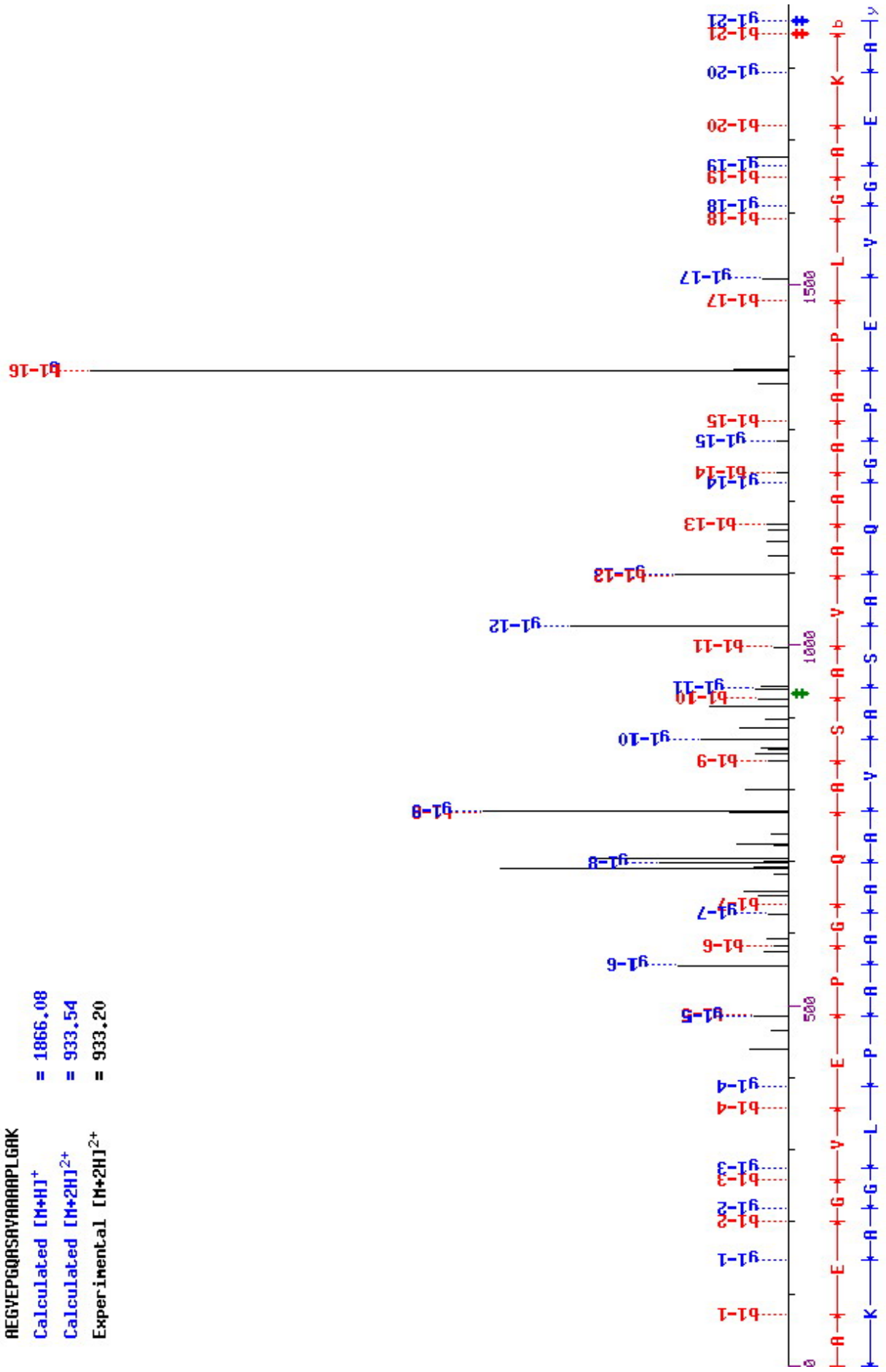
Western blot (A) and silver staining (B) of purification by Ni-NTA column. O: soluable fraction of whole cell lysate, W1: first wash, W2: second wash, W3: third wash, E1: first elution, E2: second elution. Same volume of protein solution was loaded in each lane. This result suggests that it is possible to purify the fusion product.

Small scale purification was carried out by using a Ni-NTA spin column under denaturing conditions. From the western blot and silver staining results, it was clear that the fusion product could be enriched by affinity purification. Larger scale purification was then performed and the elution fraction was concentrated and loaded on an SDS-PAGE. The band with correct molecular weight was cut out and digested in gel by trypsin. The peptides were then analyzed by Mass Spectroscopy. Four peptides with correct sequences were detected (3.4.3.3.2). This proved the identity of the expressed product as phototropin.

MAKLTSAVPVLTARDVAGAVEFWTDRLGFSRDFVEDDFAGVVRDDVTLFI
SAVQDQVVPDNTLAWVWVRGLDELYAEWSEVVSTNFRDASGPAMTEIGE
QPWGREFALRDPAGNCVHFVAEEQDENLYFQGGSHHHHHHHHHHHHAG
VPAPASQLTKVLAGLRHTFVVADATLPDCPLVYASEGFYAMTGYGPDEVL
GHNCRFLQGEGTDPKEVQKIRDAIKKGEACSVRLLNYRKDGTPFWNLLTV
TPIKTPDGRVSKFVGVQVDVTSKTEGKALADNSGVPLLVKYDHRLRDNVA
RTIVDDVTIAVEKAEGVEPGQASAVAAAAPLGAKGPRGTAPKSFPRVALDL
ATTVERIQQNFCISDPTLPDCPIVFASDAFLELTGYSREEVLGRNCRFLQGA
GTDRGTVDQIRAAIKEGSELTVRILNYTKAGKAFWNMFTLAPMRDQDGHA
RFFVGVQVDVTAQSTSPDKAPVWNKTPEEEVAKAKMGAEAASLISSALQG
MAAPTTANPWAAISGVIMRRKPHKADDKAYQALLQLQERDGKMKLMHFR
RVKQLGAGDVGLVDLVQLQGSELKFAMKTLDKFEMQERNKVARVLTESAI
LAAVDHPFLATLYCTIQTDTHLHFVMEYCDGGELYGLLNSQPKKRLKEEHV
RFYASEVLTALQYLHLLGYVYRDLKPENILLHHTGHVLLTDFDLSYSKGSTT
PRIEKIGGAGAAGGSAPKSPKKSSSKSGGSSSGSALQLENYLLLAEPSAR
ANSFVGTEEYLAPEVINAAGHGPAVDWWSLGILIFELLYGTTPFRGARRDE
TFENIIKSPLKFPSKPAVSEECRDLIEKLLVKDVGARLGSRTGANEIKSHPW
FKGINWALLRHQQPPYVPRRASKAAGGSSTGGAAFDNY

Figure 3.4.3.3.2 Mass Spectroscopy analysis of the expressed product

Fusion product was purified under denaturing condition by affinity chromatography. The elution fraction was collected and concentrated and then loaded onto SDS-PAGE. Tryptic in gel digestion was carried out afterwards. The peptides were washed out and analyzed by MS. Four peptieds were found and further sequencing analysis was carried out by MS-MS (outlined in red color), which proved the identity of the expressed product.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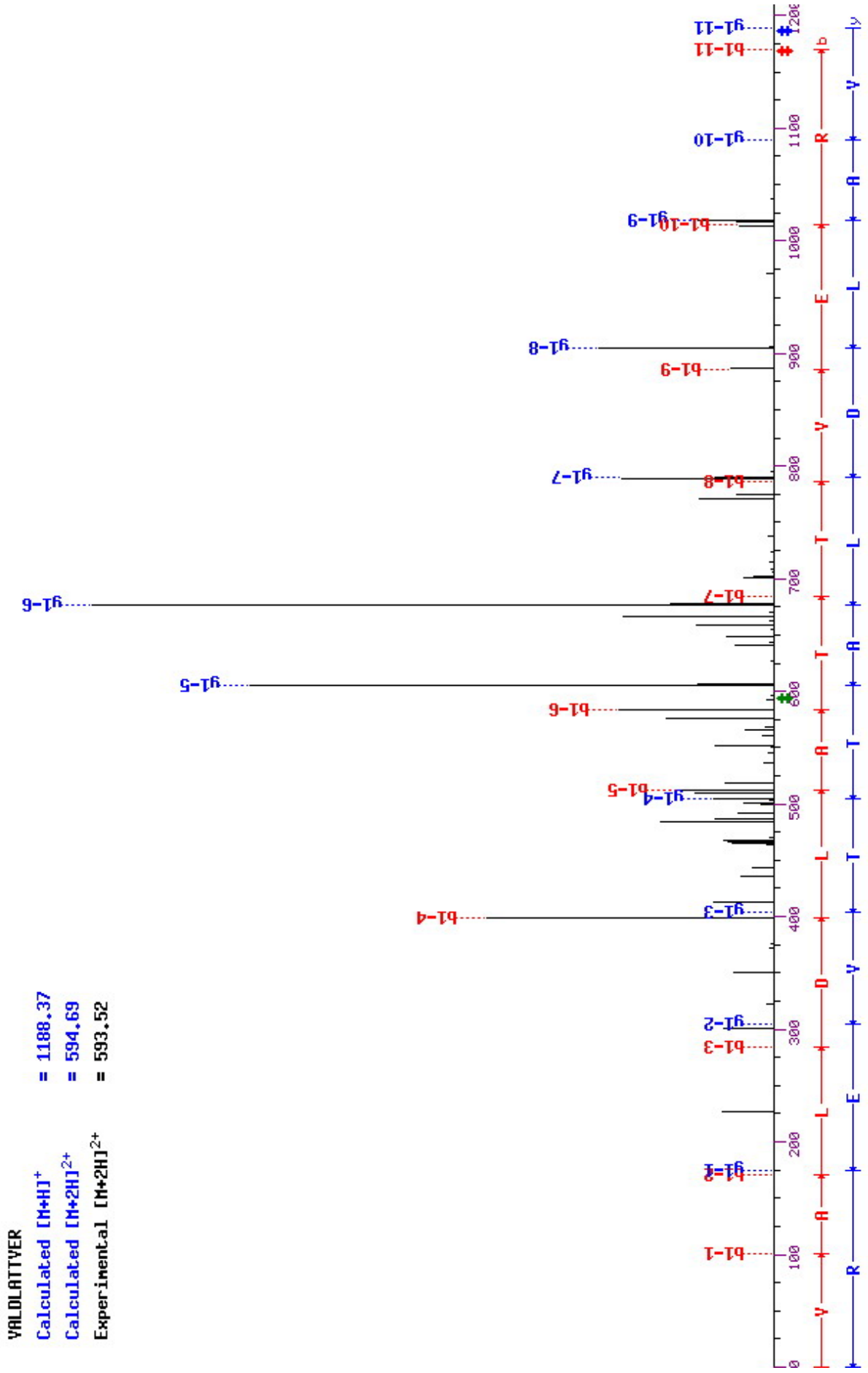
Calculated [M+2H]2+ = 933.54
Experimental [M+2H]2+ = 933.20


VALDLATTYER
Calculated [M+H]+ = 1188.37
Calculated [M+2H]2+ = 594.69
Experimental [M+2H]2+ = 593.52
b1-1
y1-1
b1-2
y1-2
b1-3
y1-3
b1-4
y1-4
b1-5
y1-5
b1-6
y1-6
b1-7
y1-7
b1-8
y1-8
b1-9
y1-9
b1-10
y1-10
b1-11
y1-11
0
100
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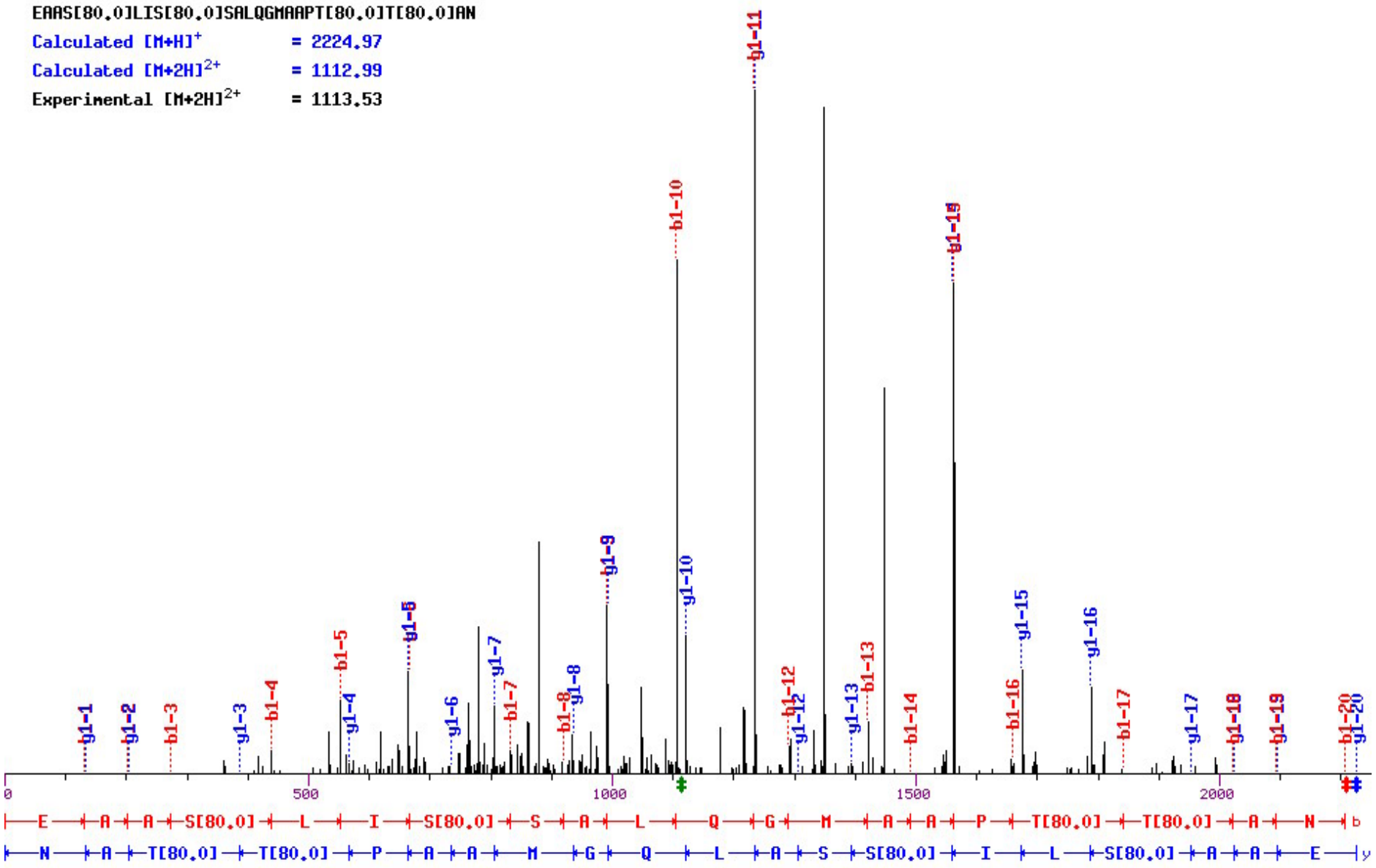
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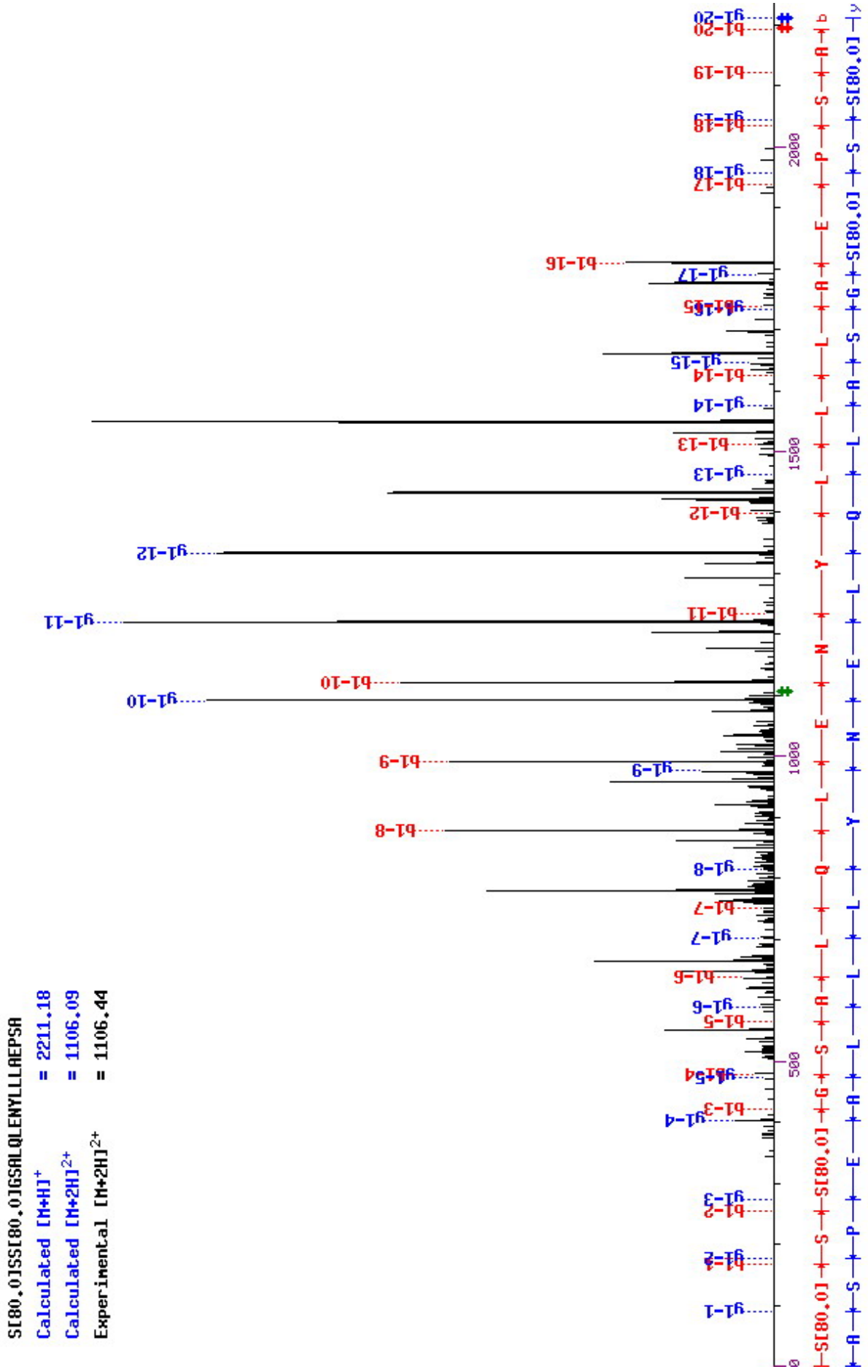
Calculated [M+H]+ = 2224.97
Calculated [M+2H]2+ = 1112.99
Experimental [M+2H]2+ = 1113.53
0
500
1000
1500
2000
E A A S[80.0] L I S[80.0] S A L Q G M A A P T[80.0] T[80.0] A N b
N A T[80.0] T[80.0] P A A M G Q L A S S[80.0] I L S[80.0] A A E y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Calculated [M+H]+ = 2211.18
Calculated [M+2H]2+ = 1106.09
Experimental [M+2H]2+ = 1106.44

3.4.4 Expression of phototropin in diatom

Although it was possible to express phototropin in *C. reinhardtii*, most of the expressed product was attached to the membrane and the expression did not bring large amount of protein in each cell. Given the exceptional strength of the *fcp* promoter (Kroger, 2001) and the possibility that the insolubility of phototropin could be caused by post-translational modification in *C. reinhardtii*, a diatom was chosen as a host to express *Chlamydomonas* phototropin. The absence of phototropin in diatom was known from the *Thalassiosira pseudonana* genome project (Armbrust et al., 2004), which was completed recently. Thus the possibility of same modification which could cause insolubility is low. The same fusion strategy that worked in *Chlamydomonas* was applied to diatoms. *Strep tag II* was placed at the C-terminus of the fusion protein.

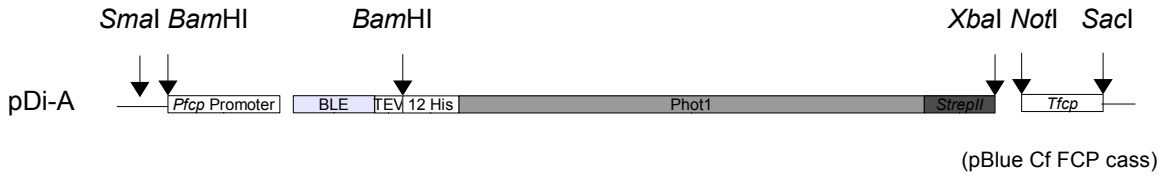


Figure 3.4.4.1 Plasmid construct for fusion expression of Ble-Phot1in Diatom

Different color boxes represent different fragments. The restriction enzyme sites are indicated above the construct. The plasmid vector is indicated at the right side of the construct. TEV, TEV protease site, *StrepII*, *Strep tag* II; *Tfcp*, *fcp* 3' UTR.

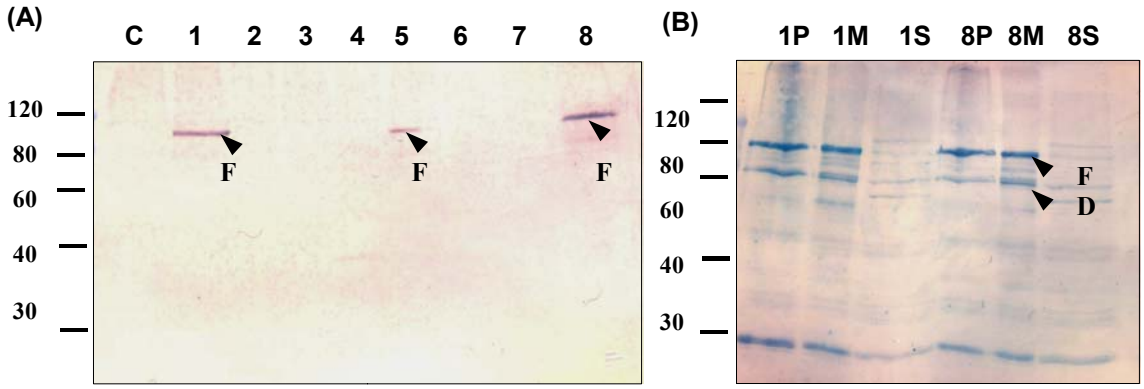


Figure 3.4.4.2 Screening of fusion expression transformants and localization of the expressed product in diatom.

(A) Screening result by western blot. Antibody raised against *Sh* Ble was used. Lane C: recipient strain used as control, Lane 1-8: different transformants. Same amount of whole cell lysate was loaded in each lane. Clone 1, 5 and 8 showed the presence of fusion expression product. (B) Transformants 1 and 8 were fractionated into low speed pellet fraction (P), membrane fraction (M) and soluble fraction(S). Lane 1P and 8P: pellet fraction, Lane 1M and 8M: membrane fraction, Lane 1S and 8S: soluble fraction. Same amount of protein (~70µg) was loaded in each lane. Although the expressed product in diatom had the correct molecular weight and degradation pattern, it mainly attached to membrane. F, full-length product (~94kD); D, degraded product (~74kD).

The transformation system for diatom is based on the method described by Apt et al.,(1996). Particle gun was used for transformation. Multiple copies of fusion expression construct were expected to be introduced into the cell. Even though, the expression level was very low (Figure 3.4.4.2). The transformants which expressed the target protein were further fractionated into low speed pellet fraction (10,000g×15min),

membrane fraction (120,000g×40min) and soluble fraction. The majority of the fusion expression product was insoluble.

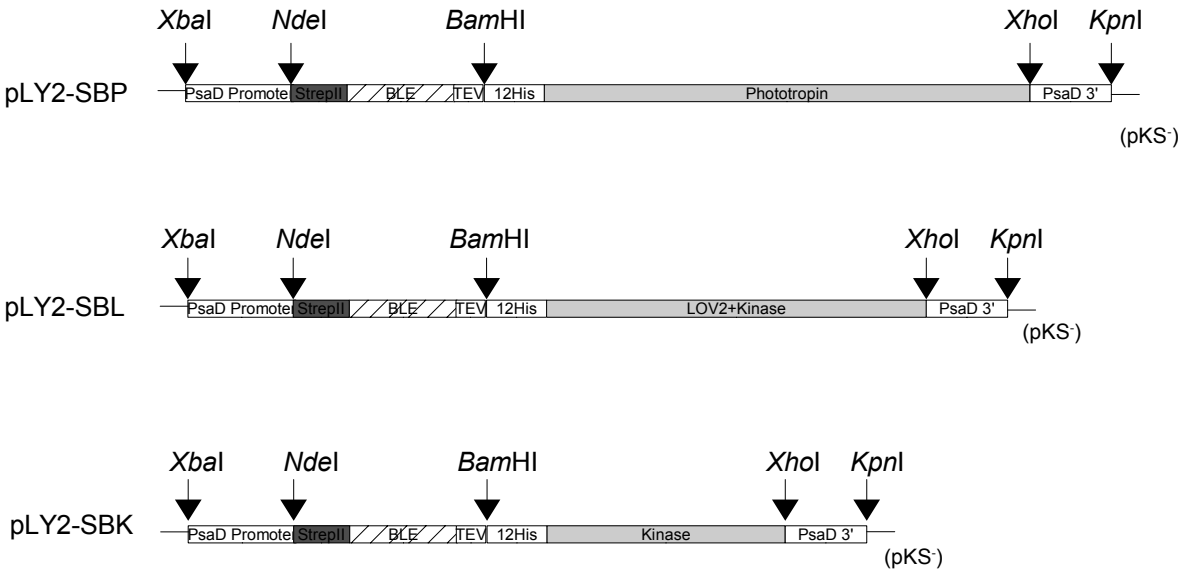
This trial of expressing phototropin only confirmed the conclusion obtained from oocyte expression experiment that the 60kD band originated from the *phototropin* gene. The degradation was in the C-terminus. However, the low expression level prevented further analysis of the fusion product in diatom.

3.4.5 Establishment of a tandem affinity purification system in *C. reinhardtii*

Till now, no interacting partner of phototropin has been identified in *Chlamydomonas*. Tandem affinity purification (TAP) is a recently developed technology to find out the interaction partners of a target protein. Several TAP tags have already been established (Puig et al., 2001; Hirano et al., 2004). With the completion of the *Chlamydomonas* genome project (Shrager et al., 2003), it is currently practical to use this technology to detect potential interaction partners of target protein in *C. reindardtii.*

3.4.5.1 Use of a directly combined *Strep* tag II at the N-terminus of the fusion products

According to the crystal structure of Ble (Dumas et al 1994), the N-terminus is sticking out of the globular fold. The first tandem affinity purification construct was designed as the *Strep* tag II was directly in the N-terminus of the Ble protein. Five different constructs were made (Figure 3.4.5.1.1). Those plasmids were transformed into strain *cw15 arg- A* and the antibody raised against *Sh* Ble was used for screening.



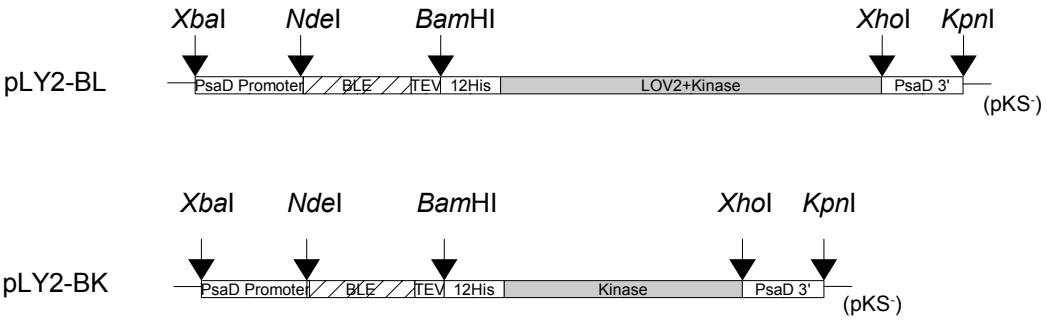


Figure 3.4.5.1.1 Schematic drawing and restriction sites of parts of the five plasmids used for expressing Ble fusion protein. Those constructs were made to test whether *Strep tag* II was suitable for being directly placed at the N-terminus of Ble. All of the constructs were flanked by the *PsaD* promoter and *PsaD* 3'UTR. *Strep tag* II is composed of eight amino acid (WSHPQFEK). The LOV2 domain used in this experiment start from the 196th amino acid of *C.r.* phototropin and Kinase domain used in this experiment start from the 362nd amino acid of *C.r.* phototropin. The restriction enzyme sites are shown above the constructs. The plasmid vector used is indicated on the right side of each construct.

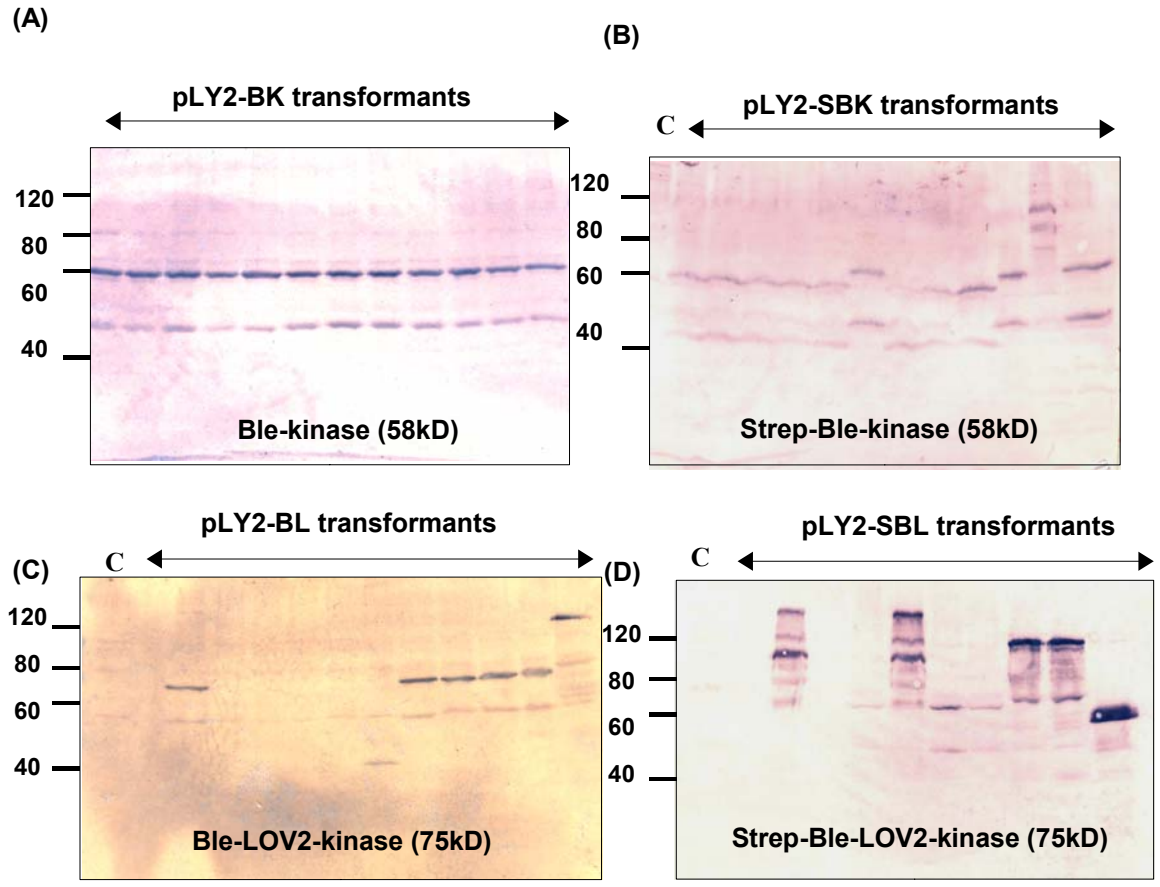


Figure 3.4.5.1.2 Comparison of transformation efficiency and products of the different fusion expression constructs with or without *Strep tag* II in the N-terminus.

(A) Western blot of screening result for pLY2-BK transformants (Ble plus Kinase domain). (B) Western blot of screening result for pLY2-SBK transformants (Ble plus Kinase domain with *Strep tag* II in the N-terminus). (C) Western blot of screening result for pLY2-BL transformants (Ble-LOV2 domain-Kinase domain). (D) Western blot of screening result for pLY2-SBL transformants (Ble-LOV2 domain-Kinase domain with *Strep tag* II in the N-terminus). Lane C, whole cell lysate of recipient strain *cw 15 arg- A*. Same amount of cells were loaded on each lane. Antibody against *Sh* Ble was used.

According to western blots of transformants from different constructs, it was found that when there was no

Strep tag II in the N-terminus of the fusion product, the transformants normally had a more or less stable expression pattern. The expressed products had the correct size and the degradation pattern was similar to that of phototropin (Figure 3.4.5.1.2 A, C). When the *Strep* tag II was directly placed at the N-terminus of the Ble protein, the expressed products did not either have the correct molecular weight or the correct degradation pattern, which suggested that *Strep* tag II affected those fusion products in protein folding or the function of Ble (Figure 3.4.5.1.2 B, D).

In pLY2-SBP, the *Strep* tag II was placed directly at the N-terminus of Ble plus full length phototropin, surprisingly, the molecular weight of the expressed product was close to the estimated value and the degradation pattern was also same as predicted (Figure 3.4.5.1.3). It seemed that the *Strep* tag II did not affect the expression of full length phototropin fusion protein. The reason was unknown. However, *Strep tag* II could not be detected with the kit provided by IBA. Those transformants were thought not suitable for purification.

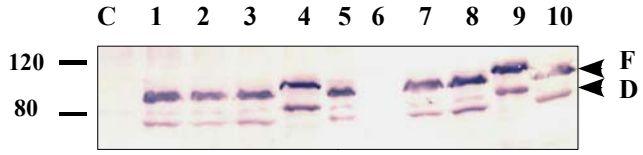


Figure 3.4.5.1.3 Western blot of screening transformants with pLY2-SBP

Western blot of screening result for pLY2-SBP. Lane C: recipient strain *cw15 arg- A*; Lane 1-10: transformants 1-10. Among those 10 clones, 8 (no.1,2,3,5,7,8,9,10) showed the correct size and proper degradation pattern. However, none of them appear positive when *Strep-Tactin AP* kit was used to detect the presence of *Strep tag* II (data not shown). F, full-length product (~94kD); D, degraded product (~74kD).

3.4.5.2 Final construct for tandem affinity purification and purification of fusion expression product

To solve the problem mentioned above, a linker sequence (ISGANGA) was added between *Strep* tag II and Ble according to Baron et al., (1992). The construct was made as shown in Figure 3.4.5.2.1.

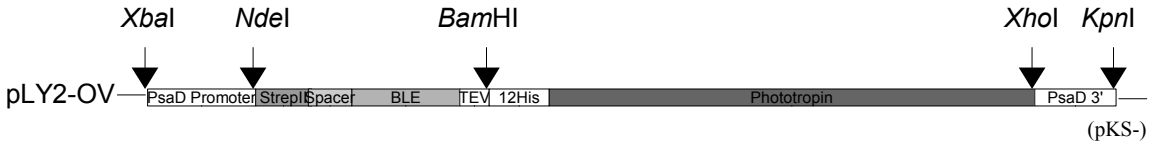


Figure 3.4.5.2.1 Final construct for fusion expression of Ble and Phototropin for tandem affinity purification.

A spacer (ISGANGA) was placed between *Strep tag* II and Ble. Different color boxes represent different fragments. The restriction enzyme sites are indicated above the construct. The plasmid vector is indicated below the construct.

pLY2-OV was transformed to *Chlamydomonas* strain *cw15 arg+* and several clones which had different

fusion products were obtained. But still, there was no positive signal when the *Strep-Tactin AP* detection kit was used (data not shown) although the antibody against Ble gave quite strong signal (Figure 3.4.5.2.2). Out of 24 transformants obtained, only two clones (No.7 and No.24) had the decent expression level of fusion expression product (Fa) with correct molecular weight (94kD) and degradation pattern. Another group of clones (No. 2, 9, 10, 11, 12) had a product (Fb) with similar degradation pattern but smaller molecular weight. They were suspected to be C-terminal truncated versions of the full length fusion product. Transformants No.9 and No.24 were picked for further analysis.

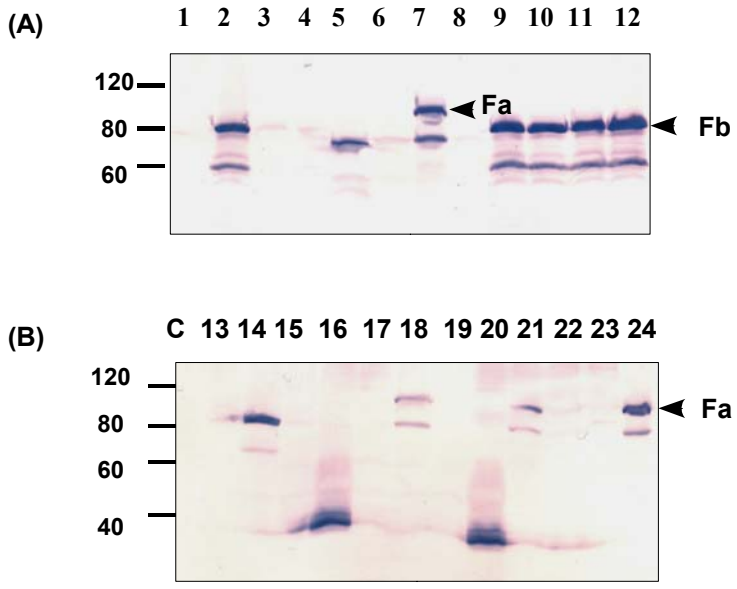


Figure 3.4.5.2.2 Screening of transformants with pLY2-OV

(A), (B): Western blot result of the screening. Antibody against *Sh* Ble was used. 24 clones were checked. Only two (No.7, 24) showed the correct molecular weight and degradation pattern. Five clones (No.2, 9, 10, 11, 12) had the fusion expressed product with a similar degradation pattern but smaller molecular weight. Lane C: recipient strain *cw15 arg- A*; Lane 1-24: whole cell lysate of transformants No.1-24. Same amount of cells were loaded in each lane. Fa, fusion product with correct molecular weight (~94kD); Fb, fusion product with smaller molecular weight (~84kD).

Cells were grown in large volume. After harvest, cells were broken with sonication and fractionated into low speed pellet, membrane fraction and soluble fraction. Soluble protein fraction were loaded onto *Strep*-Tactin Superflow Column for purification.

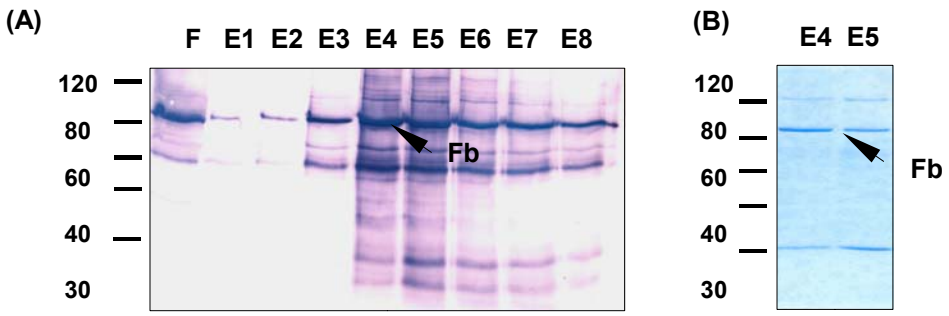


Figure 3.4.5.2.3 Purification of expressed fusion product (Fb) from strain No. 9.

(A) Western blot result of the purification. (B) Coomassie Brilliant Blue staining of the purification result. Lane F: flow through; Lane E1-E8: Elution fraction 1-8. Same volume of sample was loaded onto each lane. On the western blot and Coomassie Brilliant Blue staining result, it was shown that the truncated version of fusion product was purified. The arrows are pointing to the fusion product (Fb) which had smaller molecular weight (~84kD) on western blot and CBB stained gel.

Purification of 84kD product from the transformant No. 9 was shown as in Figure 3.4.5.2.3. In the Coomassie Brilliant Blue staining result (Figure 3.4.5.2.3 B), it was clear that the target protein was enriched in lane E4 and E5. Western blot result (Figure 3.4.5.2.3 A) showed that target protein was also enriched in lane E4, E5 after purification. According to the marker in both results, it was concluded that the band in the Coomassie Brilliant Blue staining result was the target protein.

Purification of the full length fusion product (94kD) from the transformant No.24 was shown in Figure 3.4.5.2.4. According to western blot detected with the antibody against *Sh*-Ble (Figure 3.4.5.2.4 A), target protein was enriched in fraction E3. The antibody against Phot1 LOV1 domain (Figure 3.4.5.2.4 B) only detected phototropin in the flow through fraction and target protein in fraction E3. This confirmed that the purified protein contained the phototropin part. The band of purified target protein was visible by Coomassie Brilliant Blue staining (Figure 3.4.5.2.4 C). According to the western blot, it was found that not only the expressed fusion products could be purified from both No.9 and No.24, the C-terminal degraded version of the two product could also be purified (Figure 3.4.5.2.3 A, Figure 3.4.5.2.4 A).

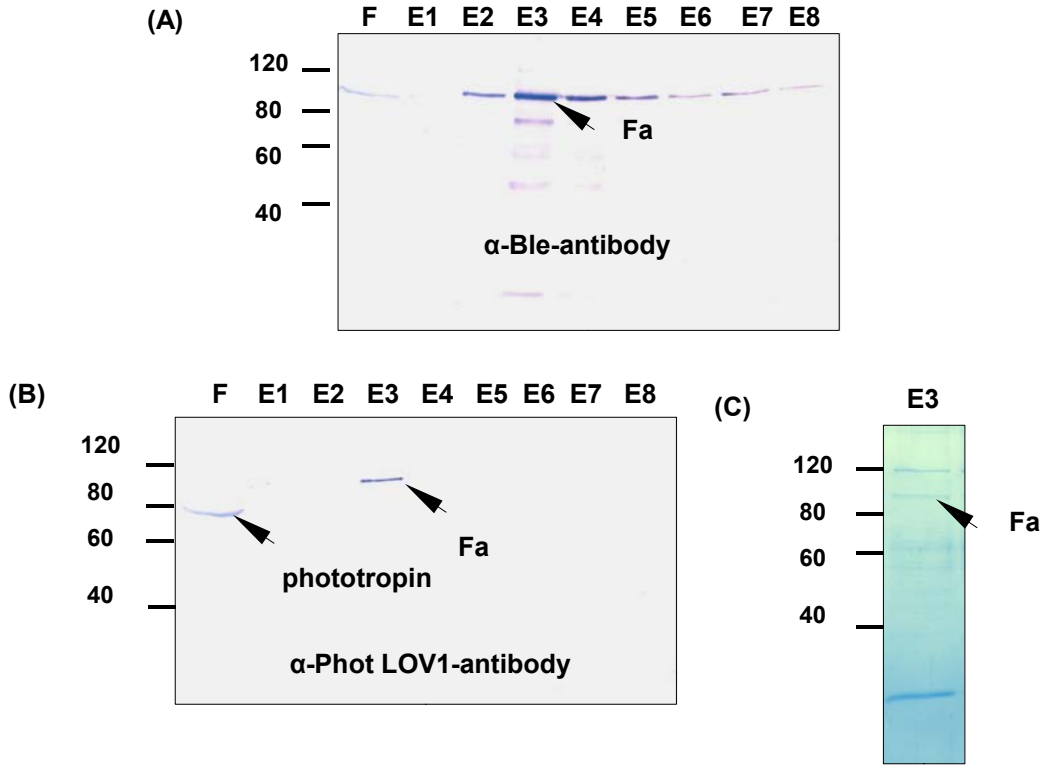


Figure 3.4.5.2.4 Purification of full-length fusion product from transformant No.24

(A) Western blot of purification result by using antibody against *Sh* Ble. (B) Western blot of purification result detected by antibody raised against phototropin LOV1-domain. (C) Coomassie Brilliant Blue staining of purification result. Lane F: flow through; Lane E1-E8: Elution fraction 1-8. In lane F to E8, same volume of sample was loaded in each lane. Fa, full-length fusion product (~94kD).

To test the purified protein's identity, the bands that were stained by Coomassie Brilliant Blue (Figure3.4.5.2.3 B, Figure 3.4.5.2.4 C) were cut out and digested with trypsin. The obtained peptides were analyzed for MS and MS-MS. In the MS analysis result of No.9, the identified peptides covered around 17.5% of the full length product (Figure 3.4.5.2.5) and MS-MS sequencing result of the selected peptide was correct. Peptides from No. 24 covered around 36.7% of the full length product (Figure 3.4.5.2.6). The selected peptide sequencing result was also correct.

MWSHPQFEKISGANGAMAKLTSAVPVLTARDVAGAVEFWTDRLGFSRDF
VEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVWVRGLDELYAEWSEVV
STNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQDENLYFQG
GSHHHHHHHHHHHHAGVPAPASQLTKVLAGLRHTFVVADATLPDCPLVY
ASEGFYAMTGYGPDEVLGHNCRFLQGEGTDPKEVQKIRDAIKKGEACSVR
LLNYRKDGTPFWNLLTVTPIKTPDGRVSKFVGVQVDVTSKTEGKALADNS
GVPLLVKYDHRLRDNVARTIVDDVTIAVEKAEGVEPGQASAVAAAAPLGAK
GPRGTAPKSFPRVALDLATTVERIQQNFCISDPTLPDCPIVFASDAFLELTG
YSREEVLGRNCRFLQGAGTDRGTVDQIRAAIKEGSELTVRILNYTKAGKAF
WNMFTLAPMRDQDGHARFFVGVQVDVTAQSTSPDKAPVWNKTPEEEVA
KAKMGAEAASLISSALQGGMAAPTTANPWAAISGVIMRRKPHKADDKAYQA
LLQLQERDGKMKLMHFRRVKQLGAGDVGLVDLVQLQGSELKFAMKTLDK
FEMQERNKVARVLTESAILAAVDHPFLATLYCTIQTDTHLHFVMEYCDGGE
LYGLLNSQPKKRLKEEHVRFYASEVLTALQYLHLLGYVYRDLKPENILLHH
TGHVLLTDFDLSYSKGSTTPRIEKIGGAGAAGGSAPKSPKKSSSKSGGSS
SGSALQLENYLLLAEPSARANSFVGTEEYLAPEVINAAGHGPAVDWWSLG
ILIFELLYGTTPFRGARRDETFENIIKSPLKFPSKPAVSEECRDLIEKLLVKDV
GARLGSRTGANEIKSHPWFKGINWALLRHQQPPYVPRRASKAAGGSSTG
GAAFDNY

Figure 3.4.5.2.5 MS result: Sequence coverage of transformant No.9. The high-lighted sequences were found in MS analysis.

MWSHPQFEKISGANGAMAKLTSAVPVLTARDVAGAVEFWTDRLGFS
RDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVWVRGLDELYAE
WSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQ
DENLYFQGGSHHHHHHHHHHHHAGVPAPASQLTKVLAGLRHTFVVA
DATLPDCPLVYASEGFYAMTGYGPDEVLGHNCRFLQGEGTDPKEVQ
KIRDAIKKGEACSVRLLNYRKDGTPFWNLLTVTPIKTPDGRVSKFVGV
QVDVTSKTEGKALADNSGVPLLVKYDHRLRDNVARTIVDDVTIAVEKA
EGVEPGQASAVAAAAPLGAKGPRGTAPKSFPRVALDLATTVERIQQN
FCISDPTLPDCPIVFASDAFLELTGYSREEVLGRNCRFLQGAGTDRGT
VDQIRAAIKEGSELTVRILNYTKAGKAFWNMFTLAPMRDQDGHARFFV
GVQVDVTAQSTSPDKAPVWNKTPEEEVAKAKMGAEAASLISSALQG
MAAPTTANPWAAISGVIMRRKPHKADDKAYQALLQLQERDGKMKLM
HFRRVKQLGAGDVGLVDLVQLQGSELKFAMKTLDKFEMQERNKVAR
VLTESAILAAVDHPFLATLYCTIQTDTHLHFVMEYCDGGELYGLLNSQ
PKKRLKEEHVRFYASEVLTALQYLHLLGYVYRDLKPENILLHHTGHVL
LTDFDLSYSKGSTTPRIEKIGGAGAAGGSAPKSPKKSSSKSGGSSSG
SALQLENYLLLAEPSARANSFVGTEEYLAPEVINAAGHGPAVDWWSL
GILIFELLYGTTPFRGARRDETFENIIKSPLKFPSKPAVSEECRDLIEKL
LVKDVGARLGSRTGANEIKSHPWFKGINWALLRHQQPPYVPRRASK
AAGGSSTGGAAFDNY

Figure 3.4.5.2.6 MS results: Sequence coverage of transformant No.24. The high-lighted sequences were found by MS analysis.

MS/MS Fragmentation of **GLDELYAEWSEVVSTNFR** **from transformant No. 9**
MONOISOTOPIC mass of neutral peptide (Mr): 2113.9956
Ions Score: 112 **Matches (Bold Red):** 38/116 fragment ions using 37 most intense peaks

#	Immon.	a	a*	a^0	b	b*	b^0	Seq.	y	y*	y^0	#
1	30.0344	30.0344			58.0293			G				18
2	86.0970	143.1184			**171.1133**			L	2057.9820	2040.9554	2039.9714	17
3	88.0399	258.1454		240.1348	**286.1403**		268.1297	D	1944.8979	**1927.8714**	1926.8874	16
4	102.0555	387.1880		369.1774	**415.1829**		397.1723	E	**1829.8710**	1812.8444	1811.8604	15
5	86.0970	**500.2720**		482.2615	**528.2669**		510.2564	L	**1700.8284**	1683.8018	1682.8178	14
6	136.0762	663.3354		645.3248	**691.3303**		673.3197	Y	**1587.7443**	1570.7178	1569.7338	13
7	44.0500	734.3725		716.3619	**762.3674**		744.3568	A	1424.6810	1407.6545	1406.6704	12
8	102.0555	863.4151		845.4045	**891.4100**		873.3994	E	1353.6439	1336.6173	1335.6333	11
9	**159.0922**	1049.4944		1031.4838	**1077.4893**		1059.4787	W	**1224.6013**	**1207.5748**	1206.5907	10
10	60.0449	1136.5264		1118.5158	1164.5213		1146.5107	S	**1038.5220**	1021.4954	1020.5114	9
11	102.0555	1265.5690		1247.5584	1293.5639		1275.5533	E	**951.4900**	934.4634	933.4794	8
12	72.0813	1364.6374		1346.6268	1392.6323		1374.6217	V	**822.4474**	805.4208	804.4368	7
13	72.0813	1463.7058		1445.6952	1491.7007		1473.6902	V	**723.3790**	706.3524	705.3684	6
14	60.0449	1550.7378		1532.7273	1578.7327		1560.7222	S	**624.3105**	**607.2840**	606.3000	5
15	74.0606	1651.7855		1633.7750	1679.7804		1661.7699	T	**537.2785**	520.2520	519.2680	4
16	87.0558	1765.8284	1748.8019	1747.8179	1793.8234	1776.7968	1775.8128	N	**436.2308**	**419.2043**		3
17	120.0813	1912.8969	1895.8703	1894.8863	1940.8918	1923.8652	1922.8812	F	**322.1879**	305.1614		2
18	129.1140							R	**175.1195**	158.0929		1

MS/MS Fragmentation of **DASGPAMTEIGEQPWGR** **from transformant No. 9**
MONOISOTOPIC mass of neutral peptide (Mr): 1800.8101
Ions Score: 81 **Matches (Bold Red):** 68/115 fragment ions using 55 most intense peaks

#	Immon.	a	a*	a^0	b	b*	b^0	Seq.	y	y*	y^0	#
1	88.0399	88.0399		**70.0293**	116.0348		98.0242	D				17
2	44.0500	**159.0770**		141.0664	**187.0719**		**169.0613**	A	1686.7909	1669.7644	1668.7804	16
3	60.0449	246.1090		228.0984	274.1039		**256.0933**	S	1615.7538	1598.7273	1597.7433	15
4	30.0344	303.1305		285.1199	**331.1254**		**313.1148**	G	1528.7218	1511.6953	1510.7112	14
5	**70.0657**	400.1832		382.1727	**428.1781**		**410.1676**	P	**1471.7003**	1454.6738	1453.6898	13
6	44.0500	471.2203		453.2098	499.2152		**481.2047**	A	1374.6476	1357.6210	1356.6370	12
7	104.0534	602.2608		584.2503	**630.2557**		612.2452	M	**1303.6105**	1286.5839	1285.5999	11
8	74.0606	703.3085		685.2979	**731.3034**		713.2928	T	**1172.5700**	1155.5434	1154.5594	10
9	102.0555	832.3511		814.3405	**860.3460**		842.3354	E	1071.5223	1054.4958	1053.5117	9
10	**86.0970**	945.4351		927.4246	**973.4301**		955.4195	I	**942.4797**	925.4532	924.4692	8
11	30.0344	1002.4566		984.4460	1030.4515		1012.4410	G	**829.3957**	812.3691	811.3851	7
12	102.0555	1131.4992		1113.4886	1159.4941		1141.4835	E	**772.3742**	755.3477	**754.3636**	6
13	**101.0715**	1259.5578	1242.5312	1241.5472	1287.5527	1270.5261	1269.5421	Q	**643.3316**	**626.3051**		5
14	**70.0657**	1356.6105	1339.5840	1338.6000	1384.6054	1367.5789	1366.5949	P	**515.2730**	**498.2465**		4
15	**159.0922**	1542.6898	1525.6633	1524.6793	1570.6848	1553.6582	1552.6742	W	**418.2203**	**401.1937**		3
16	30.0344	1599.7113	1582.6848	1581.7007	1627.7062	1610.6797	1609.6957	G	**232.1410**	215.1144		2
17	129.1140							R	**175.1195**	158.0930		1

MS/MS Fragmentation of **GLDELYAEWSEVVSTNFR** from transformant No. 24
MONOISOTOPIC mass of neutral peptide (Mr): 2113.9956
Ions Score: 65 **Matches (Bold Red):** 41/116 fragment ions using 40 most intense peaks

#	Immon.	a	a*	a^0	b	b*	b^0	Seq.	y	y*	y^0	#
1	30.0344	30.0344			58.0293			G				18
2	**86.0970**	143.1184			**171.1133**			L	2057.9820	2040.9554	2039.9714	17
3	88.0399	258.1454		240.1348	**286.1403**		268.1297	D	1944.8979	1927.8714	1926.8874	16
4	102.0555	**387.1880**		369.1774	**415.1829**		397.1723	E	**1829.8710**	1812.8444	1811.8604	15
5	**86.0970**	**500.2720**		482.2615	**528.2669**		510.2564	L	1700.8284	1683.8018	1682.8178	14
6	136.0762	663.3354		645.3248	691.3303		673.3197	Y	1587.7443	1570.7178	1569.7338	13
7	44.0500	734.3725		716.3619	762.3674		744.3568	A	1424.6810	1407.6545	1406.6704	12
8	102.0555	863.4151		845.4045	891.4100		873.3994	E	1353.6439	1336.6173	1335.6333	11
9	**159.0922**	1049.4944		1031.4838	1077.4893		1059.4787	W	**1224.6013**	1207.5748	1206.5907	10
10	60.0449	1136.5264		1118.5158	1164.5213		1146.5107	S	**1038.5220**	1021.4954	1020.5114	9
11	102.0555	1265.5690		1247.5584	1293.5639		1275.5533	E	951.4900	934.4634	933.4794	8
12	**72.0813**	1364.6374		1346.6268	1392.6323		1374.6217	V	**822.4474**	805.4208	804.4368	7
13	**72.0813**	1463.7058		1445.6952	1491.7007		1473.6902	V	**723.3790**	706.3524	705.3684	6
14	60.0449	1550.7378		1532.7273	1578.7327		1560.7222	S	**624.3105**	607.2840	606.3000	5
15	74.0606	1651.7855		1633.7750	1679.7804		1661.7699	T	**537.2785**	520.2520	519.2680	4
16	87.0558	1765.8284	1748.8019	1747.8179	1793.8234	1776.7968	1775.8128	N	**436.2308**	**419.2043**		3
17	120.0813	1912.8969	1895.8703	1894.8863	1940.8918	1923.8652	1922.8812	F	**322.1879**	305.1614		2
18	**129.1140**							R	**175.1195**	158.0929		1

MS/MS Fragmentation of **DVAGAVEFWTDR** **from transformant No. 24**

MONOISOTOPIC mass of neutral peptide (Mr): 1364.6360
Ions Score: 72 **Matches (Bold Red):** 23/76 fragment ions using 33 most intense peaks

#	Immon.	a	a^0	b	b^0	Seq.	y	y*	y^0	#
1	88.0399	88.0399	**70.0293**	116.0348	98.0242	D				12
2	**72.0813**	**187.1083**	169.0977	**215.1032**	197.0926	V	**1250.6169**	1233.5904	1232.6064	11
3	44.0500	258.1454	240.1348	**286.1403**	268.1297	A	**1151.5485**	1134.5220	1133.5380	10
4	30.0344	315.1668	297.1563	**343.1617**	325.1512	G	1080.5114	1063.4849	1062.5008	9
5	44.0500	**386.2039**	368.1934	**414.1989**	396.1883	A	1023.4899	1006.4634	1005.4794	8
6	**72.0813**	485.2724	467.2618	**513.2673**	495.2567	V	952.4528	935.4263	934.4423	7
7	102.0555	614.3149	596.3044	**642.3099**	624.2993	E	853.3844	836.3579	835.3739	6
8	120.0813	761.3834	743.3728	**789.3783**	771.3677	F	**724.3418**	707.3153	706.3313	5
9	**159.0922**	947.4627	929.4521	**975.4576**	957.4470	W	**577.2734**	**560.2469**	559.2629	4
10	74.0606	1048.5103	1030.4998	1076.5053	1058.4947	T	**391.1941**	374.1676	373.1836	3
11	88.0399	1163.5373	1145.5267	1191.5322	1173.5216	D	**290.1464**	273.1199	272.1359	2
12	129.1140					R	**175.1195**	158.0930		1

4. Discussion

4.1 Locaization and distribution of phototropin in *Chlamydomonas reinhardtii*

In higher plants, phototropin is in charge of controlling the movements such as phototropism, chloroplast relocation and stomatal opening so that the plant can make the best use of sun light while avoiding the harmful effect which may be caused by too strong sun light (Briggs and Christie, 2002). As a single-celled organism, *C. reinhardtii* exhibits phototactic behavior. It has been suggested to be triggered by microbial-type rhodopsin (Foster et al., 1984; Lawson et al., 1991; Takahashi et al., 1991). It seems that the blue light receptor phototropin has little effect in helping cell to select the best place for growth. And till now, phototropin has only been reported to be involved in the sexual life of *C.reinhardtii* (Huang et al., 2002; Huang et al., 2003).

In Huang et al., (2002), quantitative competitive RT-PCR and western blot were applied to determine the level of phototropin in different growing stages. In that experiment, vegetative cells, cells grown in darkness, gametes directly generated from vegetative cells, pregametes, and gametes generated form pregametes had been tested. Among those different kinds of cells, both the phototropin level and *phototropin* mRNA level were the lowest in vegetative cells, but phototropin levels in other cell types are similar (Huang et al., 2002). Till now the function of phototropin in vegetative cells has not been reported. Is phototropin unimportant in vegetative cells? The answer is no. In this thesis, vegetative cells of different strains grown under same light conditions were tested. The levels of phototropin in these cells were different (Figure 3.1.1.2 A). Then vegetative cells from three different strains grown under same conditions were fractionated. It was discovered that the phototropin distribution also varied in these cells (Figure 3.1.1.2 B). The level of phototropin in vegetative cells is very delicately regulated, which suggest that slight change in its level may bring harmful damage to the cell. Vegetative cells grown in light or darkness were fractionated. The distribution and level of phototropin was different under these two conditions (Figure 3.1.2.1 D). Light is one factor that is in charge of regulating phototropin level and distribution. But obviously, light is not the only factor. The other factors are to be discovered.

For the first time, it was shown that phototropin existed as a soluble protein in *Chlamydomonas*. It was reported to be an insoluble protein in *Chlamydomonas* in Huang et al., (2002), Huang et al., (2004). It was shown in this thesis that no matter in exponential stage of vegetative cells, stationary stage of vegetative cells or in gametes, phototropin existed as an soluble protein as well as a membrane attached protein (Figure 3.1.2.1) Although the soluble fraction only counts for small amount of total soluble protein, it opened a chance for purification. From the amino acid sequence analysis, it is known that phototropin has no hydrophobic

region which allows integration into the membrane. In *Chlamydomonas*, phototropin could attach to the membrane by some adapter protein or by binding to a second messenger such as DAG (diacylglycerol). Or, it could be covalently modified by a fatty acid. Some detergents and chaotropic salts have been tried to wash it off the membrane, but the efficiency was very low (data not shown). This suggests that other methods are required to solubilize the protein.

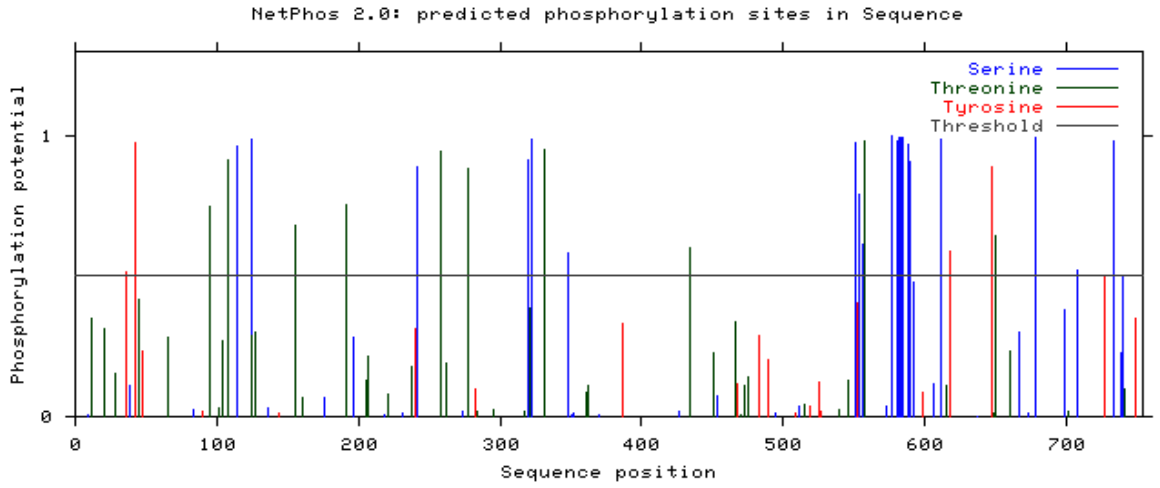


Figure 4.1.1 Phosphorylation prediction of *C. r.* phototropin (NetPhos 2.0 Server).
According to the prediction, there are 20 Ser, 10 Thr and 4 Tyr phosphorylated in phototropin after excitation. The phosphorylation will increase the molecular weight ~2.7 kD.

In *Arabidopsis*, phototropin was always considered as a membrane associated protein (Sakamoto and Briggs, 2002). Upon excitation by light, phototropin accumulates in certain regions of cytoplasm. It has not been shown yet which region the excited phototropin enters (Sakamoto and Briggs, 2002). It has also been shown for mustard (Knieb et al., 2004) that in darkness 100% phototropin seems to be attached to the membrane fraction. When activated by blue light illumination, around 20% of membrane attached phototropin entered the cytoplasm (Knieb et al., 2004). The mobilities in SDS-PAG of activated phototropin also changed, it appeared to be 2-3kD larger on western blot result, which could be caused by phosphorylation (Knieb et al., 2004). In *C.reinhardtii*, it seems to be a different case. Soluble phototropin has lower apparent molecular weight than its counterpart in the membrane fraction (Figure 3.1.1.1). In darkness, there are more phototropin remaining soluble compared with the cell grown in light conditions (Figure 3.1.2.1 D). The soluble phototropin could be the unphosphorylated form of phototropin while the phosphorylated form is attached to the membrane. According to Netphos 2.0 prediction, there are 32 potential sites in the *Chlamydomonas* phototropin that could be phosphorylated. Phosphorylation will add ~2-3 kD to the molecular weight (Figure 4.1.1). The molecular weight difference between membrane attached phototropin and soluble phototropin is not so pronounced in endogenous phototropin. It is much more obvious in the phototropin fusion expression product (Figure 3.4.3.1.1).

The C-terminus of phototropin belongs to protein kinase C super family (Huala et al., 1997). The behavior of *Chlamydomonas* phototropin is somewhat very similar to PKC, which is a soluble cytosolic protein and catalytically inactive without hormone stimulation. For PKC, a rise in cytosolic Ca^{2+} level causes it to bind to the plasma membrane where it gets activated by membrane-associated DAG (Newton 1996). In *Arabidopsis*, blue light can activate voltage-dependent and calcium-permeable channels and cause Ca^{2+} influx (Stoelzle et

al., 2003). In *Chlamydomonas*, it can be easily test whether the increase in Ca^{2+} concentration causes phototropin to attach to the plasma membrane.

In *Arabidopsis*, phototropin shows a light induced down-regulation pattern. Transformants which expressed phototropin-GFP fusion protein was used in that experiment. Fluorescence was decreased after 24h of illumination with blue light. And the immunoblot result also showed that the decrease in fluorescence was not caused by the bleaching of GFP but by the down-regulation of the phototropin level (Sakamoto and Briggs, 2002). In *Chlamydomonas*, phototropin is also down regulated by light. Its degradation pattern is different in cells grown in high light and in darkness (Figure 3.1.3.1 A). Under high light conditions, more phototropin degradation product was seen. Short strong illumination did not increase the degradation of full length phototropin (Figure 3.1.3.1 B). It was found that similar to *Arabidopsis*, the down-regulation process took more than 24 hours. A light gradient was then applied to identical portions of cells grown in the same preculture. The degradation pattern of phototropin was shown to be related to the light intensity. The degradation product of phototropin remains attached to the plasma membrane and was a stable component of the cell, suggesting that the degradation may happen after the activation of phototropin. However, in strain *cw15 arg- A* and *CC477* grown under same light conditions, the degradation pattern of phototropin was different (Figure 3.1.1.2). It seems that there could be other factors that regulate the degradation.

In *Arabidopsis*, there are two phototropins: Phot1 and Phot2. Both Phot1 and Phot2 mediate phototropism, chloroplast relocation and stomatal opening. Although Phot1 and Phot2 have redundant functions, they seem to work under different light intensities (Sakai et al., 2001; Briggs and Christie 2002; Liscum, 2002). Phot1 and Phot2 protein abundance are correlated to the *Phot1* and *Phot2* mRNA abundance. *Phot1* mRNA abundance in etiolated seedlings appears to decrease when exposed to long-term continuous light (Sakamoto and Briggs, 2002). In contrast, *Phot2* mRNA is increased in etiolated seedling after exposure to light (Sakai et al., 2001; Jarillo et al., 2001; Sakamoto and Briggs, 2002).

Since there is only one phototropin gene in *Chlamydomonas* (Huang et al., 2002), the second band seen in western blot could only be a phototropin degradation product or an alternative RNA splicing product. Since the degradation process takes more than 24 hours and in *Chlamydomonas,* there are already examples of mRNA alternative splicing (Fuhrmann et al., 2003), it is possible that there are one phot1 gene but two mRNA products derived from it. The difference in abundance of the two bands could result from the abundance change of mRNA level under different light conditions. In cress (*Lepidium sativum)* seedling extract, a second band (~20 kD smaller than full length phototropin) also showed a reduced mobility on SDS-PAG after illumination (Knieb et al., 2004). The author assumed it to be a degradation product or a second phototropin.

Phototropin fused with *Sh* Ble in the N-terminus was expressed in *Chlamydomonas* and diatom. Phototropin

was successfully expressed in oocyte (Chapter 3.4). There were no introns in the expression constructs but the degradation pattern still exists. It is less likely that the same splicing machinery happened in different organisms. In the future, RT-PCR should be applied to find out whether there are two kinds of *phototropin* RNAs in *C. reinhardtii*.

According to western blot results (Figure 3.1.1.2) and fusion expression results (Figure 3.4.3.1.1), it was clear that the degradation occurred in the C-terminus of phototropin. It could be a protection mechanism for *Chlamydomonas* against strong blue light.

4.2 Silencing of phototropin in *C.reinhardtii*

RNAi technology is a popular tool to silence target genes, however, the enzymes which attend the process have not yet been identified in *Chlamydomonas*. The reason to use RNAi technology to reduce the phototropin level in *Chlamydomonas* lies in the fact that it is not yet practical to knock out of *Chlamydomonas* endogenous gene by homologous recombination. Recently, homologous recombination carried out by single strained DNA shed the light on the possibility of using homologous recombination to knockout endogenous gene in *Chlamydomonas* (Zorin et al., 2004). However, trials to knockout *phototropin* gene in *Chlamydomans* by this method were unsuccessful in this work.

Fuhrmann et al., (2001) put forward the hypothesis that *Chlamydomonas* had the RNAi mechanism and also proved the assumption by silencing a *Chlamyopsin* gene (*cop*). The best construct for silencing shown in that paper was composed of a leading genomic fragment fused to a reverted cDNA counterpart under the control of the *cop* promoter. The authors believed that after transcription and RNA splicing, a perfect hairpin structure would form and trigger the RNAi process. The function of those introns in the leading genomic piece was to prevent recombination potential of the hairpin structure during cloning in *E.coli* and to assist in maintaining the expression level of transgene similar to that of the authentic *cop* gene. *cw15 arg- A* was used as recipient strains. It seems that in *cw15 arg- A*, the RNAi construct works perfectly. Half of the transformants obtained by co-transformation with marker plasmid pSI103 showed a significant reduction in their opsin content (Fuhrmann et al., 2001).

A similar strategy was applied to silence the phototropin gene in this thesis. An artificial genomic fragment was used in place of the authentic genomic fragment of phototropin. The construct seemed to work well in strain *cw15 arg- A.* 12.5% of the transformants showed a reduction of phototropin of around 95%. However, this RNAi construct did not work properly in other strains. Although intensive transformation and screening had been carried out, only one clone with a less pronounced reduction level of phototropin was found. It seems that different strains may have different sensitivities to the same RNAi construct, which could be caused by the integration position of the construct. Since the selection marker and RNAi construct were in different plasmids, co-transformation efficiency may also affect the number of positive clones.

The efficiency of RNAi silencing was different under different growth condition. It was found for the transformant *C4*, the best silencing conditions was low light conditions (Chapter 3.3). According to the mechanism of RNAi, the reduction level should be quite stable under all kinds of conditions since RNAi is a self-amplifying process. The different promoter used instead of the original phototropin promoter could also be an explanation for the phenomenon. The combination of phototropin promoter with piece of phototropin

genomic fragment could help to adjust RNAi construct transcripts level more close to phototropin mRNA, i.e., the levels of the two newly synthesized mRNAs could be almost synchronized. Successful silencing has been achieved in this way (Fuhrmann et al., 2001, Huang et al., 2003).

Whether or not to use 3' UTR in the RNAi construct is a interesting problem. The 3' UTR is important to a mRNA, it decides not only the ending of transcription and the tailing but also the delivery, stability and localization of mature mRNA. A successful example has shown that it was not necessary to have a 3' UTR in the RNAi construct for the *Chlamyopsin* gene (Fuhrmann et al., 2001). As in the formal result obtained with strain *cw15 arg- A*, the RNAi construct used in this thesis also worked nicely (Figure 3.2.2.1). But in other strains, the strategy seems not so efficient, which suggests that lacking of 3' UTR could be the reason. Several studies already showed that RNAi process was restricted to the cytoplasm (Zeng and Cullen, 2002; Kawassaki and Taira et al., 2003). Lacking 3' UTR could also cause low efficiency in ending of transcription and delivering mRNA out of the nucleus. Since piece of foreign DNA is randomly integrated into *C. reinhardtii* genome (Kindle et al., 1989), the only identified *CC32pab1mt(+)* transformant could result from the case that the RNAi construct happened to locate in front of a 3' UTR of one *Chlamydomonas* gene. Without 3' UTR, tailing of mRNA could be less efficient. Normally, mRNA without polyA tail will enter deadenylation-dependent pathways. It would be degraded either by 3' to 5' exonucleolytic decay or 5' to 3' exonucleolytic decay. In either way, siRNA would not be created. Thus the hairpin RNA would not work properly.

The use of artificial genomic DNA instead of authentic genomic DNA could be another explanation for low silencing efficiency. There are two known RNAi interference pathways. One is the short interfering RNA (siRNA) pathway which starts with double-stranded RNA and form siRNA by Dicer. The other pathway is through miRNA (micro interfering RNA) (Pasquineli et al., 2002). When there is imperfect hairpin RNA structure, Dicer could also digest them into short RNA pieces (miRNA). Those miRNAs are believed to bind to sites that have partial sequence complementarity in the target mRNA. miRNA can direct the cleavage of a perfectly complementary target RNA. When miRNA binds partially complementary target RNA, it blocks the translation (Cerutti, 2003). In the experiment described in this thesis, the artificial genomic piece of phototropin was composed of several small pieces and conjugated with different restriction enzyme sites. After post-transcriptional modification, the two introns would be removed but restriction enzyme sites would still remain, which caused the product to be an imperfect hairpin RNA. After cleavage, RISC guided by small pieces of RNA which would bind to target RNA with few unpaired base would not cleave the target RNA. Thus the self-amplification of RNAi is limited. The different performance of same RNAi construct in different strains could be caused by many reasons. To obtain more phototropin silencing strains, new RNAi constructs need to be made.

In the obtained RNAi construct transformants, both full length phototropin level and truncated version of phototropin level got reduced. The fact further proved that those two proteins originated from one gene. In case that these two proteins carry out different function, it would be interesting and challenging to study the differences.

4.3 Mating assay

In *Chlamydomonas*, blue light was found to be closely related with the sexual life (Weissig, 1991). In *Chlamydomonas* gametogenesis, the first condition required is nitrogen starvation. When vegetative cells incubated in darkness are deprived of nitrogen in the medium, they develop into pregametes. The second signal is blue light, after which pregametes acquire mating competence and change into gametes. Blue light is also necessary for maintaining the mating competence (Huang and Beck, 2003). Since the relationship between phototropin and gametogenesis has been clearly demonstrated in Huang and Beck, 2003, in this thesis, the focus was placed on the involvement of phototropin in zygote germination.

As mentioned in Gloeckner and Beck, (1995), light is required to trigger meiosis and germination of C. *reinhardtii* zygotes. Huang and Beck (2003) further put forward the possibility of phototropin involvement in this process. Reduction of the zygote germination rate was found in the offspring of phot- strain (Huang and Beck, 2003). However, the experiment protocol used by the authors of the two papers mentioned above is complicated and difficult to handle. This difficulty limited the usage of that method. The complexity of the process increased the possibility of man-made mistakes. The distinction between vegetative, unmated gametes and zygotes is not absolutely accurate. To overcome these drawbacks, a new assay was established in this thesis.

It was found in this thesis that deficiency in phototropin results in a delay of zygote germination under moderate light conditions. Intensive light input can overcome the lag caused by the low level of phototropin. In the interpretation of the result obtained from the mating assays, three factors are necessary to be placed under consideration. The first is how closely *pab1* gene and *aphVIII* gene are linked in the *Chlamydomonas* genome i.e. are they on the same or different chromosomes. The second is the level of phototropin in transformant *C4*'s offspring. The third is the presence of vegetative diploid cells.

Transformant *C4* and control strain *G5* were generated by transformation with *aphVIII* gene separately. *aphVIII* gene integrated into the *Chlamydomonas* genome randomly. Although there are 17 linkage groups in *Chlamydomonas* genome, there is still the possibility that in *C4* or in *G5*, *aphVIII* fell into the same linkage group as the *pab1* gene. The chance is high that *aphVIII* and *pab1* are unlinked in both strains (16/17 × 16/17). The possibility is 2× 16/17 × 1/17 that in one strain the two genes were linked and in the other strain the two genes were in different linkage groups. It is also possible that in both strains, the two gene were linked (1/17 × 1/17). In this assay, *aphVIII* and *pab1* are regarded as unlinked.

In those zygotes produced from the mating of *C4* and *CC124*, there were two sets of chromosomes present in the nucleus. Whether the RNAi construct was transcriptionally active was unknown. In the vegetative cells

produced from those zygotes, some contained the RNAi construct in their genome, some did not. How could the result be analyzed? The resolution was simple. As introduced in the introduction part, RNAi machinery takes place in cytoplasm. RdRp can use single-stranded siRNA as primer and target mRNA as template to synthesize double-stranded RNA. Those double-stranded RNAs can enter the cycle again. RNAi has a self-amplifying process. During zygote formation and meiosis, the siRNA left in the cytoplasm would trigger the process. Thus, the zygotes formed from *C4* and *CC124* were regarded as Phot1-.

The presence of vegetative diploid cells is another factor that should be considered in the data analysis. Under normal laboratory conditions, about 1-5% of mated gamete pairs of *C. reinhardtii* do not form meiotic zygotes. They form vegetative diploids (Harris, 1989; Ebersold, 1967) instead. The difference between germinated zygotes and vegetative diploid cells is that the diploid cells begin to divide shortly after mating whereas meiotic zygotes require several days to mature before germination (Harris, 1989). Thus the colonies of vegetative diploids came out earlier and were much bigger than those formed by germinated zygote. Those big colonies were not counted.

It is possible to remove the impact of vegetative diploids. On TAP medium, vegetative diploids can grow independently of the light conditions. Thus their number should be similar in high light condition, middle light condition and low light condition. They appeared as big colonies on plates. The number could just be removed from the colony numbers obtained under high light and middle light conditions.

By comparing the zygote germination situations between low light condition and middle light conditions or high light conditions, the conclusion that light is necessary for zygote germination could be drawn. By comparing the offspring of the transformant and the control strain in middle condition, it is concluded that phototropin is an important factor for zygote germination. Under certain light conditions, those zygotes with higher phototropin level would germinate faster. By comparing the offspring of the RNAi construct transformant in middle light and hight light conditions, the conclusion could be reached that strong light could compensate the lack of phototropin and accelerate zygote germination.

4.4 Expression and purification of phototropin

As an eukaryotic single-celled organism, *C. reinhardtii* has several advantages in expressing recombinant proteins over prokaryotic systems such as *E. coli*. 1.) With bioinformatic analysis, a total of 34 *Chlamydomonas* open reading frames, potentially encoding members of the five major chaperone families and the GrpE and Cpn10/20 co-chaperones, have been discovered (Schroda, 2004). Thus all the newly synthesized proteins have a milder folding surrounding compared with *E. coli*, which may greatly help the foreign peptides especially those from other eukaryotic systems to fold into their nature conformation. 2.) *Chlamydomanas* has its protein modification system, which could not only help to stabilize the protein conformation but also increase the protein's resistance against protease. 3.) The growth medium for this alga is quite simple and cheap. Cultures can be easily upscaled. This allows large cultures to be used as starting material when expression level of protein is low. But the drawbacks for *Chlamydomonas* as an expression system are also quite obvious. 1.) Foreign gene randomly integrates into the nuclear genome, when *Chlamydomonas* is transformed (Rochaix, 1995). This random integration could cause some unknown problem such as truncated expression or some other deleterious effect on the cell growth. 2.) *Chlamydomonas* cells can turn off the expression of foreign genes autonomously. The mechanism for such kind of silencing is still unknown (Cerutti et al., 1997). 3.) In most cases, cell wall deficient strains such as *cw15 arg- A* were chosen for transformation. Although they are easy to transform, it is difficult to grow them in large volume. They are not able to stand high pressure or shearing force and break in large volume culture, which could cause contamination easily.

The expression level of recombinant protein is decided by the transcriptional efficiency, degradation speed of mRNA, translation efficiency and speed of protein degradation. Thus, the strength of promoter is of great importance. The *AR* promoter (*HSP70A* promoter plus *RbcS2* promoter) is a very strong promoter (Goldschmidt-Clermont and Rahire, 1986; Kindle, 1998; Schroda et al., 2000). In this thesis, *AR* promoter was not chosen to drive the expression of *Ble* gene fused with phototropin cDNA. It has been clearly demonstrated that insertion of the first intron of the *RbcS2* gene into the *Sh* Ble coding sequence increased expression level around 30-fold when compared with *RbcS2* promoter alone. The transcription driven by *RbcS2* promoter need the aid from *RbcS2* introns. Another strong promoter, *PsaD* promoter was chosen for driving the nucleic expression of the fusion product. The *PsaD* gene encodes an abundant protein of the Photosystem I complex and does not contain any introns in its coding sequence. The absence of introns in the *PsaD* gene suggests that the transcription driven by this promoter does not need additional information. The strength of the *PsaD* promoter is stronger than *RbcS2* promoter alone and similar with *RbcS2* plus first intron (Fisher and Rochaix, 2001).

Even with *PsaD* promoter, the trials to express *phototropin* cDNA met continuous failure (pLYM-D, pLYM-E in Chapter 3.4.3). Most of *Chlamydomonas* nuclear genes contain several small introns in the coding sequences and intronic sequences appear to have an important role in the regulation of gene expression (Silflow, 1998). Using genomic sequences in complementation of mutants seems to be more efficienct than their cDNA counterpart (Diener et al., 1993; Auchincloss et al., 1999; Perron et al., 1999; Boudreau et al., 2000). Studies of transgenes in higher eukaryotes, including plants, have demonstrated a positive role for introns in gene expression. In some cases, intron splicing appears to be required for efficient nuclear export or stability of transcripts (Huang and Gorman, 1990; Rose and Last, 1997). The introns of the phototropin gene could be important for transcription. It has not been tried to put several introns in the over-expression

construct. Obviously, such attempt may result in some improvement (Eichler-Stahlberg, 2005).

Although the fusion expression strategy may not be the best way to express phototropin, it is the only method that currently works. Ble protects the cell by binding zeocin to prevent it from cutting DNA (Drocourt et al., 1990; Gatignol et al., 1988). To work efficiently, Ble proteins need to form dimers (Dumas et al., 1994). The presence of Strep tag II in its N-terminus without spacer seemed to hinder Ble part in the fusion product to form dimer (Figure 3.4.5.1.2).

From an widely accepted point of view, Ble protein needs to enter the nucleus to bind zeocin, thus the fusion expression products with Ble are required to be soluble. The fusion strategy with Ble has been tried in *Chlamydmonas* (Fuhrmann et al., 1999), where GFP was directly linked to the C-terminal of Ble. The fusion product was localized only in the nucleus (Fuhrmann et al., 1999). This finding suggests that there should be a nuclear localization signal (NLS) within the Ble protein sequence. The majority of phototropin attaches to the membrane, but a small portion of the protein remains soluble. The percentage of soluble protein in the expressed fusion product is similar to that of the original phototropin. This suggests that the mechanism which causes the insolubility of those two proteins could be same or closely related. The localization of the expressed fusion product is not determined in detail, and the mechanism of the fusion product helping the cells survive in presence of zeocin is unknown. The possible explanation could be the following: newly synthesized soluble fusion protein managed to enter the nucleus guided by the NLS of Ble. Those proteins remained soluble in nucleus and helped to protect the cell; during cell division, those proteins were released to the cytoplasm. When the new nucleus was formed, those soluble fusion proteins entered the nucleus again. Another explanation could be that the expressed protein formed a matrix attached to the plasma membrane and prevented zeocin entering the nucleus. However, further test such as fusing a GFP to the C-terminus of phototropin or isolating and fractionating nucleus should be carried out. Ble fused with kinase or LOV2 plus kinase domain could also be expressed in the same way, but almost 100% of the expressed product remained insoluble (data not shown), which support the matrix assumption.

The fusion expression strategy would be very useful for future studies of protein functional analysis in *Chlamydomonas*. And the method seems to have several advantages: 1.) Since the marker protein fused to the target protein, co-transformation is not required. 2.) The transformants are easy to screen. In Chapter 3.4.2, out of 80 clones, 66 showed the correct expression pattern, around 82% of the transformants were positive. 3.) The expression level of target protein could be regulated. Ble is a zeocin binding protein, the higher concentration of zeocin is used in the medium, the higher level of over-expression product is required for the cell. Thus, in a certain range, increased concentrations of the antibiotics can be used to identify those transformants which have high level of target protein. 4.) Transformants are not easily silenced. Normally introduced genes in *Chlamydmonas* are unstable in their expression level (Cerutti et al 1997). In this fusion strategy, those silenced cells could be easily killed by putting the transformants under selection pressure again. However, the safest way currently to keep *Chlamydomonas* transformants is still to freeze them in liquid nitrogen.

The fusion constructs encoding Ble -Kinase and Ble-LOV2-Kinase resulted in a low expression levels. Recently, Mastuoka and Tokutomi studied GST fused with kinase domain, GST fused with LOV2 - kinase construct and GST fused with LOV1-LOV2-kinase. When the kinase domain was expressed without LOV2 domain, kinase domain was always active. When LOV2 plus the kinase domain was expressed, the kinase domain got activated under low light conditions. When LOV1-LOV2-kinase was expressed, the kinase domain got activated only under high light conditions (Figure 4.4.1) (Mastuoka and Tokutomi, 2005).

Although there is still debate whether LOV1 domain or LOV2 domain gets activated first, it was clearly demonstrated the presence of LOV1 and LOV2 domain helped to keep the kinase domain inactive in darkness or under low light conditions. This finding could explain the low expression level of Ble-kinase protein and Ble-LOV2-kinase protein in *Chlamydomonas*. Ble-kinase was permanently active and Ble-LOV2-kinase was easily activated. Thus, the over-expressed product might be harmful for the cells. They can only exist in low concentration.

It also could explain the failure of trials to over-express phototropin directly. Excess amount phototropin might be toxic to the cells under moderate light conditions. The expression level of recombinant phototropin is low. Thus, unconspicuous improvement in phototropin level would be hardly detected. The reason that the fusion expression product could be detected was due to the high recognition efficiency of anti *Sh* Ble antibody. The trials to express *C.r.* phototropin directly may be carried out in the future. Genomic PCR or RT-PCR should be applied in screening for the transformants. *Strep tag* II may be directly placed in the N-terminus or C-terminus of phototropin cDNA. The expressed product could be purified in the same way in Chapter 3.4.6.2.

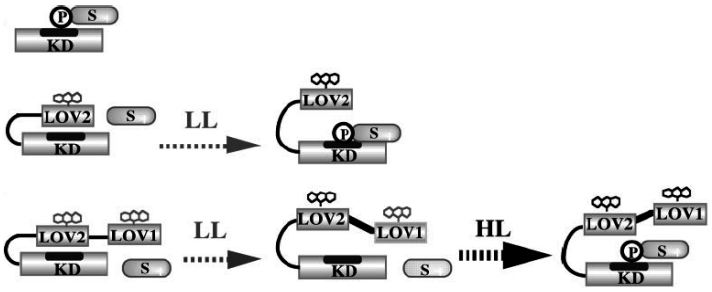


Figure 4.4.1 Schematic illustration of the roles of LOV1 and LOV2 domains in light regulation of substrate phosphorylation by Kinase domain in *A.t.* Phot2. P: phosphate; S, substrate; LL, low light conditions; HH, high light conditions (Mastuoka and Tokutomi, 2005).

It has been found that phototropin was very delicately regulated (Chapter 3.1). This regulation is consistent with the importance for growth and development of the cell. There are reports about success in expressing phototropin in *phot1* null mutant (Sakamoto and Briggs, 2002) or *phot1phot2* null mutant (Onodera et al., 2005) in *Arabidopsis.* However, success in over-expressing phototropin has not been reported. It is unknown whether excessive phototropin could be toxic for the cells. The reason for the success of fusion expression could be that *Sh*-Ble protein in the N-terminus of the fusion product blocked some proper functions of phototropin and made the cells less sensitive to the over expressed product.

An interesting transformant was found which only expressed part of the fusion product with better solubility. The missing of phototropin C-terminus may make the fusion product more hydrophilic and easy to purify. In the sequence analysis, around 50 aminoacids in the C-terminus of phototropin seem to be not necessary for the kinase activity (Huang et al., 2002), which suggest that this short fragment could be either modified or interacting with other proteins to form aggregates. Expression the 'truncated' version of the fusion product in *Chlamydomonas* might be a good approach to prepare material for protein crystallization.

As described in Chapter 3.1, in the cell wall deficient strain *cw15 arg- A*, under the same growth conditions, the amount of soluble phototropin is much less than that in the bald strain *CC477*. Different genetic background of the two strains could be the reason for that. *cw15 arg- A* is not suitable for growing of large scale cultures because of its fragile cell wall. In the future, bald strains are a good choice for expressing soluble phototropin for purification.

5. Materials and methods

5.1 Methods about handling with *C. reinhardtii* (buffer recipes included)

5.1.1 Growing *C. reinhardtii* in liquid culture

C. reinhardtii could be grown in liquid culture in Erlenmeyer flasks. In this thesis, 100ml Erlenmeyer flask with ~50ml culture medium was commonly used for growing *Chlamydomonas*. To prevent *Chlamydomonas* from precipitating or sticking together, the liquid culture in Erlenmeyer flasks were shaken or stirred continuously. The shaking speed was around 120rpm and stirring speed was around 200rpm. According to different requirement, different light intensities were applied to *Chlamydomonas* cultures. High light condition was ~9-10W/m^2, middle light condition was ~4W/m^2 and low light condition was less than 1W/m^2. The growing tempeature was 25ºC.

For screening large numbers of transformants, 96-well plates and 24-well plates were used for growing single *Chlamydomonas* clones. Those plates were transparent and made of plastic. They were placed under middle light condition (~4W/m^2) and were kept still.

For light gradient experiment, a home made light filter was used to create a light intensity gradient (20W/m^2, 14.2W/m^2, 11W/m^2, 9.4W/m^2, 5.2W/m^2, 3W/m^2, 1.6W/m^2, 0.71W/m^2, 230μW/m^2, 160μW/m^2, 95μW/m^2). 24 well plates were used for growing cells. They were kept still and were blown with an electric fan to get rid of the heat caused by the strong light intensity.

Both HSA (high salt acetate) and TAP (Tris-Acetate-Phosphate) were used as media for vegetatively growing *Chlamydomonas*. For strain 806 and cw2, HSAS was used. For strain *cw15 arg- A* or *CC48 arg-*, TAPA, TAPAYZ, TAPATYZ or TAPAP was used. For wild type strain, *CC124mt(-)*, *CC125mt(+)*, *CC620mt(+)*, *CC621mt(-)* and bald strain *CC477*, *CC478* and *CC479*, TAP or HSA was chosen as growing media. For dark growing culture, TAPTY was used. For *CC32pab1mt(+)*, TAPP, TAPY, TAPYP or TAPPP was used.

TAP(Tris-Acetate-Phosphate)

NH_4Cl	7.5 mM
$MgSO_4$	0.4 mM
$CaCl_2$	0.34 mM
K_2HPO_4	0.54 mM
KH_2PO_4	0.46 mM
NaAc	5 mM
Tris/Cl pH 7.0	20 mM
Hunter's trace elements	0.1%

Hunter's trace elements

$ZnSO_4 \cdot 7H_2O$	22 g
H_3BO_3	11.4 g
$MnCl_2 \cdot 4H_2O$	5.06 g
$FeSO_4 \cdot 7H_2O$	4.99 g
$CoCl_2 \cdot 6H_2O$	1.61 g
$CuSO_4 \cdot 5H_2O$	1.57 g
$(NH_4)_6Mo_7O_{24} \cdot 2H_2O$	1.1 g
EDTA	50 g

Dissolve in 1 liter water

TAPA(Tris-Actate-Phosphate-Arginine)
TAP medium with 50mg/l L-arginine

TAPAYZ(Tris-Actate-Phosphate-Arginine-Yeast extract-Zeocin)
TAPA medium with 10mg/l zeocin and 0.3%Yeast extract

TAPATYZ(Tris-Actate-Phosphate-Arginine-Trypton-Yeast extract-Zeocin)
TAPAYZ medium with 0.2% Bacto-Tryptone

TAPAP(Tris-Actate-Phosphate-Arginine-Paromomycine)
TAPA medium with 10mg/l paromomycine

TAPTY(Tris-Actate-Phosphate-Arginine-Trypton-Yeast extract)
TAP medium with 0.2% Bacto-Tryptone and 0.3%Yeast extract

TAPY(Tris-Actate-Phosphate-Yeast extract)
TAP medium with 0.3% Yeast extract

TAPYP(Tris-Actate-Phosphate-Yeast extract-Paromomycine)
TAPY medium with 10mg/l Paromomycine

TAPPab(Tris-Acetate-Phosphate-PAB)
TAP medium with 50mg/l PAB (p-aminobenzoic acid)

TAPP(Tris-Acetate-Phosphate-Paromomycine)
TAP medium with 10mg/l paromomycine

TAPPP(Tris-Acetate-Phosphate-PAB-Paromomycine)
TAPP medium with 10mg/l Paromomycine

HSA(High Salt Acetate)

NH_4Cl	9.3 mM
$MgSO_4$	0.81 mM
$CaCl_2$	0.1 mM
K_2HPO_4	6.2 mM
KH_2PO_4	6.8 mM
NaAc	15 mM
Hunter's trace elements	0.1% (v/v)

HASS(High Salt Acetate Sorbitol)

HAS medium with 250mM Sorbitol

5.1.2 Growing *Chlamydomonas reinhardtii* on Agar

Standard bacteriological petri plates(150×15mm) were used for growing *Chlamydomonas* transformants. 1.5% agar was added to the media mentioned above. Those plates are placed in 25ºC with 4W/m^2 light.

For keeping strains, agar slants in test tubes were used. Those agar slants contain 1.5% to 2% agar and TAPTY medium. Screw-capped test tube were used. Stock culture were kept in 18ºC under low light (<1W/m^2).

5.1.3 Gametogenesis

Gametes were generated by resuspending vegetative cells in nitrogen minimal medium (NMM). Vegetative cells were collect by centrifugation at 2,500 rpm× 5min. The pellet was resuspended in NMM and centrifugated at 2,500 rpm× 5min again. Then the cells were resuspended in NMM at a density of ~1×10^7 cells/ml and were placed under continuous light for 48 hours. To prepare pre-gametes, vegetative cells were collected and washed the same way as above. The the pellet was resuspended in NMM at a density of ~1×10^7 cells/ml and was incubated in darkness continuously for 48 hours. Gametes could also be prepared from pregamete by incubating pregametes under continuous illumination for 2 hours.

NMM (Nitrogen minimal medium)

$MgSO_4$	0.81 mM
$CaCl_2$	0.1 mM
K_2HPO_4	6.2 mM
KH_2PO_4	6.8 mM

5.1.4 Isolation of Flagella (pH Shock Protocol)

Cells grown in 2-liter culture were harvested by centrifugation (2,500rpm×5min) and resuspended in 100ml 10mM Tris buffer, pH7.8. Then the cells were collected at 2,500rpm for 5 min again and resuspended in 100ml 10mM Tris buffer, pH7.8. The cells were centrifugated again and the pellet was resuspended in 100ml 7% sucrose-10mM Tris buffer, pH7.8, at 4ºC. All the steps after this were carried out at 4C and it was essential to work quickly. Sedimented cells left in a pellet rapidly lysed, releasing their internal constituents and contaminating the final preparation of flagella.

The suspension of cells in 7% sucrose-Tris was vigorously stirred with a magnetic stirrer and 910µl 0.5N acetic acid was quickly added to rapidly decrease pH to 4.5. After 90 sec, 90-100% of the cells deflagellated. 1080µl 0.5N KOH was then added to the suspension to raise pH to 7.8.

For separation of the flagella, 15-ml aliquots were transferred to chilled 50-ml conical polycarbonate centrifuge tubes. 20ml of 25% sucrose-10mM Tris buffer was then placed under the 15ml aliquot of cell suspension. The tubes were centrifuged at 2000g for 11 min. The flagella were left in the 7% sucrose-10mM Tris buffer layer. The 7% sucrose-10mM Tris buffer layer was then collect and merged.

To remove any remaining cell bodies, 20ml portion of flagella suspensions were underlayered with 4 ml 25% sucrose-10mM Tris buffer as above and centrifuged for 9 min at 1800g. The supernatant was collected and centrifuged at 27,000 g for 20 min. The sedimented flagella were then obtained.

5.1.5 Flagella fractionation

To obtain soluble fraction of flagella, ice-cold F buffer(pH7.4) was used to resuspend flagella pellet. The buffer volume was also around 1.5 times of the initial packed cell volume. F buffer was composed of 30mM HEPES, 5mM $MgSO_4$, 1mM dithiothreitol, 0.5mM Na_2EDTA, 25mM KCl, 0.5% polyethylene glycol (20,000 Da). It was FN buffer without detergent. 1mM phenylmethysulfonyl chloride (PMSF) was added to minimize proteolysis. Repeated freezing in liquid nitrogen and thawing in 37°C incubator were applied to the resuspension. Then the resuspension was centrifuged at 100,000g for 40 min. The supernatant was considered as the soluble fraction of flagella. The pellet was considered as the insoluble fraction of flagella.

5.1.6 Preparation of Gamete Autolysin

Wild-type strain *CC124mt(-)* and *CC125mt(+)* were pre-grown in 500-ml liquid phototrophic culture for 2 days to a final cell density of ~7×10^6 cells/ml. Cells were then harvested by centrifugation at ~2000rpm for 5 min. The pellet was washed once with NMM and then resuspend in NMM at a density of 2×10^6 cells/ml. The cultures were illuminated with continuous light for 24 hours. Gametes were then harvested and resuspended at a density of ~4×10^7 cells/ml in NMM. The two mating types were mixed and mated for 1 hour.

After mating, cells were collected by centrifugation at 3000g for 5 minutes. The supernatant fraction was collected and spun at 18,000g for 10min. Then the supernatant was lyophilized and resuspended in a small volume of NMM for use.

5.1.7 Transformation of *Chlamydomonas*

5.1.7.1 Transformation of cell-wall-deficient strain (Glass bead method)

Cells were grown to mid-log phase (1-2×10^6cells/ml) in TAPTY medium under continuous bright light (~$4W/m^2$). The cells were then harvested by spinning at 2,500g for 5min. The centrifugation should be carried out at room teperature. The cells were resuspended in fresh TAPTY mediumby gentle pipetting to a concentration of 2×10^8cells/ml. 300µlof the cell suspension was transferred into 2ml eppendorf tube containing 300mg of 0.4mm diameter glass beads. 1µg of linearised DNA containing the construct for transformation and 100µl 20% PEG6000 (sterile) were added. If selection marker was not present in the same plasmid, 1ug of linearised DNA which contained selection marker was added as well. The mixture was mixed by Vortex for 15sec. The cells were then transferred to a 100ml Erlenmeyer flask with 50ml TAPTY medium and grown overnight (~18 hours) under continuous light at 25°C. The next day, the cells were pelleted by centrifugation for 5 min at 2,500 rpm. The pellet was gently resuspended in ~0.5ml TAPTY

medium and then plated on TAPTY 1.5% agar with 10 µg/ml zeocin.

5.1.7.2 Transformation of wild type cell

Autolysin was used in the transformation. Cells were grown to mid-log phase (1-2×10^6cells/ml) in TAPTY medium under continuous bright light (~4W/m^2). Then autolysin was added in to the culture and the flask was kept in darkness for 30-45 min. The cells were then harvested by spinning at 2,500g for 5min. The rest steps were same as in 5.1.7.1 .

5.1.8 Mating Assay

In this thesis, one metabolic deficient strain *CC32pab1mt(+)* with promomycin resistance was chosen as one mating partner and one wild type strain *CC124mt(-)* was chosen as another mating partner.

Pre-grown culture of both mating types were grown to mid-log phase under middle light condition (~4W/m^2) in TAPTY medium. Cell density was measured. About 5×10^6 cells of *CC124mt(-)* were inoculated in fresh TAP medium. Around 5×10^6 cells of *CC32pab1mt(+)* transformants C4 and G5 were inoculated in fresh TAPPab medium. All the cultures were incubated in low light condition (~1W/m^2) at 25°C for 5-7 days until cell density reached 2-3×10^6 cells/ml.

The cells were then harvested and the cell numbers of different strains were counted. The pellet was washed once with NMM and resuspended in NMM at 1×10^7 cells/ml. The cell suspension was kept in low light condition for 2 days (48 hours) at 25°C.

The cells were harvested again and cell number of different strain was counted. 5×10^6 cells of C4 and 5×10^6 cells of CC124mt- was mixed, and 5×10^6 cells of control strain G5 and 5×10^6 cells of CC124mt- was mixed. Those mixtures were kept in 2 ml sterile cups. The cells were spun at 1000g for 4 min and the volume was adjusted to 1ml in each mating reaction. The pellet was resuspended by vortex. Those cups were then placed in 25°C baker with light (~8W/m^2).

After one hour, the cell mixtures were centrifuged at 1000g for 4 min again and 500µl of NMM was discarded. The pellets were resuspended again and were plated on TAPP agar plates. Those plates were kept in high light condition (~9W/m^2), middle light condition (~4W/m^2) or low light condition (<1W/m^2) at 25°C. Every 7 days, cell number on each plate was checked and compared.

5.1.9 Fractionation of *Chlamydomonas* cells

Cells were grown to a density of 5×10^6 cells/ml and harvested by centrifugation at 2,500 rpm for 5 min. The pellet was then resuspended in ME buffer and sonicated 15times in ice bath. The homogenate was centrifuged at 13,000g for 15min. The pellet was composed of large organelles. The microsomal fraction was pelleted by centrifugation at 120,000g for 40 min. The supernatant after this centrifugation was regarded as cytoplasmic fraction.

ME buffer

MOPS	10mM
EDTA	1mM

Adjust pH to 6.8

5.2 Methods about handling with Diatom *Cylindrotheca fusiformis* (buffer recipes included)

5.2.1 Growing *C. fusiformis*

Diatom cells could be grown in liquid culture in Erlenmeyer flasks. In this thesis, 250ml Erlenmeyer flask with ~100ml culture medium was commonly used for growing diatom. The cells were kept in 18°C room with continuous light illumination (~10W/m^2).

Since diatom cells had a strong tendency to stick together and form big clump of cells, cell culture should be shaken vigorously. The cultures were normally shaken at 300rpm.

ASW medium (Artificial Sea Water medium) was used to grow diatom. The diatom strain could either be kept in liquid culture or agar slant (2%). In either case, culture should be kept in dim light (<1W/m^2). When strain was kept in liquid, test tube filled with 5-7cm ASW medium was used. The medium should be renewed every four weeks. For the strains kept on agar slant, the strains should be inoculated onto fresh slant every two months.

ASW-Medium

Glycylglycine	600mg
NaCl	23.4g
1M $CaCl_2$	7.5ml
2M $MgSO_4$	10ml
2M $MgCl_2$	10ml
3M KCl	2 ml
0.5M KNO_3	3 ml
100mM Na_2SiO_3	2 ml
1000 ×SE solution	1 ml
1mg/ml Thiamin	0.5ml

Dissolve in 1 liter H2O and adjust to pH8.0 with 1M NaOH. Autoclave the medium. After cooling down to room temperature, 2ml of 100mM K_2PO_4 should be added.

1000×SE solution

H_3BO_3	1.14g
$Na_2EDTA \cdot 2H_2O$	6.05g
$ZnCl_2$	620mg

$CuCl_2 \cdot H_2O$	270mg
$Na_2MoO_4 \cdot 2H_2O$	250mg
$CoCl_2 \cdot 6H_2O$	420mg
$FeCl_2 \cdot 4H_2O$	970mg
$MnCl_2 \cdot 4H_2O$	360mg

Dissolve in 1 Liter H_2O

5.2.2 Transformation of diatom cells

5.2.2.1 Adsorption of plasmid-DNA on tungsten particles (M17)

Tungsten particles should be washed before use. 10mg Tungsten particles were put in to 500µl ethanol (p.a.) and carefully resuspended. The suspension was then spun for 1 min at 4000rpm. The supernatant was discarded and 250µl ethanol (p.a.) was added. The pellet was then resuspended and the suspension was vortexed for 1-2min. The mixture was centrifuged at 4000rpm for 1 min and the supernatant was discarded. Then, the pellet was washed for three tims with 250µl strile water. Each time, the mixture was resuspended by vortex for 1-2 min. After the last centrifugation, 150µl H_2O was added to resuspend the pellet. Aliquot in 50µl portion was put in 1.5ml sterile cups.

To each 50µl aliquot of tungsten particles, 5µl 1ug/µl plasmid-DNA, 50µl 2.5M $CaCl_2$ (Sterile) and 20µl 0.1M Spermidin (free base, sterile) were added under constant vortexing. The mixture was then vortexed for 3 minutes and centrifuged for 10 second. The supernatant was discarded afterward.

The pellet was resuspended in 250µl Ethanol (p.a.) by vortexing for 1 min. Again, the suspension was centrifugated for 10 second and the supernatant was removed. The pellet was then resuspended in 50µl ethanol (p.a.). The mixture was incubated on ice until transformation.

5.2.2.2 Preparation of diatom cells

C. fusiformis cells were grown to mid-log phase (1×10^6cells/ml) when the culture appeared a light brown. The cell number was counted by using hemocytometer. For each plate, 5×10^7cells were needed. Those cells were harvested in sterile 50ml Falcon tube by room temperature centrifugation at 2,800g for 10 min. The cells were then resuspended in ~50ml ASW medium for washing. The cells were spun down again at 2800g for 10 min. The pellet was resuspended and for each plate, the volume should be ~250µl. The suspension was plated in the central region (Ø ca. 5cm) of an ASW-plate.

5.2.2.3 Shooting of cells

Before shoot cells, rupture disc and microcarriers should be washed in ethanol (p.a.) and dried in a petri dish in the clean bench. Stopping screen and microcarrier-holder were kept at 200°C for 4 hours before use. All parts of the particle gun were first washed with 70% ethanol. A microcarrier was placed into a microcarrier holder. The DNA-coated tungsten particles were resuspended and 10µl of the suspension was taken out and dropped in the central area of the microcarrier. Then the microcarrier was left in clean bench for ~10 min for drying.

The microcarrier holder was placed in the microcarrier Lauch assembly. A Rupture disk was placed in a Rupture disk holder. The ASW-plate with cell suspension was then put on the plae holder and the distance between Rupture disc and the agar plate should be ~7cm.

The particle deliver chamber was evacuated and cells were shot with He as pressure gas. The pressure

should be 1350 psi. After shooting, the pressure was returned to atomshperic pressure and the plate was removed. The cells were then kept in culture room (18°C) with continuous light illumination for 24 hours.

5.2.2.4 Selection of resistant *C. fusiformis* Cells

The ASW plate was washed once with 2ml and once with 1ml fresh ASW medium. The cell number was then counted. Then the cells were resuspended in ASW at a density of 10^7cells per 250µl ASW medium.
250µl of such cell suspension was plated on a ASWZ plate. Those plates were placed in culture room (18°C) for 7-10 days with continuous illumination (~6W/m^2). Clones of transformants could be seen afterwards.

5.3 Methods about handling with *Escherichia coli*(buffer recipes included)

5.3.1 Growing *E. coli*

The standard medium for *E. coli* growth was LB. For plasmid preparation, TB was used. In the liquid culture, the cells were grown at 37°C with shaking (250rpm). Transformants for E. coli were screened on LB agar plates with ampicillin (100µg/ml), the plates were kept in 37°C baker for clones growing.

LB

Bacto Trypton	10g
Bacto Yeast extract	5g
NaCl	10g

Disslove in 1 Liter H_2O and autoclave.

TB

Media component

Bacto Trypton	12g
Bacto Yeast extract	24g
Re-distilled glycerol	4g

Disslove in 900ml H_2O and autoclave.

Phosphate component

KH_2PO_4	2.3g
K_2HPO_4	12.5g

Disslove in 100ml H_2O and autoclave. When the media and phosphate solution are cool, combine them to

give 1 liter of TB.

5.3.2 Making competent cells of *E. coli*

A 2-3 mm diameter colony was picked from a freshly streaked SOB agar plate and inoculated in 250ml Erlenmeyer flask containing 50ml SOB medium (The cells were best streaked from a frozen stock). The culture was kept in 37°C baker overnight with continuously shaking (>180rpm).

On the next morning, the overnight culture was inoculated in 500ml Erlenmeyer flask containing 200ml SOB medium until the OD_{578} reached around 0.05. The culture was then kept in 37°C baker for around 2-3 hours till OD_{578} reached 0.3-0.4.

The cells were harvested by centrifugation at 2400 rpm for 7 min (4°C). The pellet was resuspended in 15ml Tfb I buffer(kept on ice before use). The incubation times varied for different strains. For DH5α the incubation time should be 10 min while for BL21 the incubation time should be 30 min. The suspension was spun at 2,000 rpm for 5 min (4°C). The pellet was resuspended with 2ml TfbII buffer (kept on ice before use).

Aliquot of 110µl of the cell suspension was put into 500ml PCR tube and frozen quickly in liquid nitrogen. The cells were kept in -80°C freezer and could be used at any time.

SOB-Mg

Bacto tryptone	20g
Bacto yeast extract	5g
BaCl	10 ml of a 1M stock
KCl	2.5ml of a 1M stock

Dissolve in 1 liter DDW and autoclave.

$MgCl_2$+$MgSO_4$ 2M stock

Combine 203g/l $MgCl_2 \cdot 6H_2O$ and 247g/l $MgSO_4 \cdot 7H_2O$ in DDW and sterilize by filtration through a pre-rinsed 0.2um filter unit.

SOB medium	
SOB-Mg medium	1 liter
$MgCl_2$ + $MgSO_4$	10 ml of a 2M stock

Tfb I

KAc	30mM
$MnCl_2 \cdot 2H_2O$	50mM
KCl	100mM
Glycerol	15%(v/v)

Use HAc to adjust pH to 5.8 and sterilize by filtration through a pre-rinsed 0.2um filter unit. The solution should be kept in 4°C.

Tfb II

MOPS	10mM
$CaCl_2$	75mM
KCl	10mM
Glycerol	15%(v/v)

Use NaOH to adjust pH to 7.0 and sterilize by filtration 987ewqthrough a pre-rinsed 0.2um filter unit. The solution should be kept in 4°C.

5.3.3 Transformation of *E. coli* cells

The tubes prepared from the step above was removed from the freezer and thawed on ice. DNA solution (volume < 10ul) was added. The tube was swirled gently to mix the DNA evenly with the cells. The tube was then incubated on ice for 10-30 min.

The cells were then treated with heat shock by placing the tube in a 42°C water-bath for 90 sec. The tube was chilled on ice for 1-2 min. 800µl of SOC medium was added in the tube and the tube was incubated at 37°C with moderate agitation for 30-60 min. The suspension was spread on SOB agar plate containing appropriate antibiotics. The plate was incubated at 37°C overnight. Colonies would appear in 12-16 hours.

SOC medium

SOB-Mg medium	1 litre
$MgCl_2$ + $MgSO_4$	10 ml of a 2 M stock
Glucose	10 ml of a 2 M stock

5.3.4 Storing and retrieving *E. coli* in frozen at -80°C

5.3.4.1 Preparing frozen stocks of *E. coli*

Some E. coli strain was collected by a flamed tungsten loop from an agar plate, a liquid culture or a previous frozen stock. The cells were spread on an SOB agar plate. The plate was then incubated at 37°C until 2-3mm diameter colonies formed. One colony was picked and inoculated in a test tube containing 5ml SOB medium. The tube was kept in 37°C with agitation till the cells reached the mid-log phase (OD_{550}=0.5-0.7). The culture was then 1:1 diluted with a solution composed of 60% SOB medium and 40% glycerol. The mixture was vortexed gently and aliquot of 0.5-1ml cell suspension was put in series of 2 ml cups. The cups was put into liquid nitrogen for fast freezing and then stored in -80°C.

5.3.4.2 Retrieving E. coli from -80°C frozen stocks

A tube of the frozen stock was removed from the freeze and placed on dry ice. Quickly scrape a clump of frozen stock from the surface of the frozen suspension. The frozen clump of cells was placed on an SOB agar plate and it would thaw immediately. The cells are dispersed to obtain single colony.

The plate was incubated at 37°C overnight.

5.4 Methods about handling with *X. laevi*s oocyte (buffer recipes included)

5.4.1 Microinjection of *X. laevis* oocyte and detection of target protein expression

mRNA of target protein was prepared by RNA synthesis kit (Ambion). 10 ng of mRNA was injected into one mature oocyte. Those injected oocytes were kept in 18°C with constant agitation.

Three days later, the injected oocytes were harvested, each in one 500µl PCR tube. 50µl of HbA buffer was added. The oocytes were broken by pipetting. The tubes were centrifugated at 200g for 1 min and the supernatant was transferred to another clean tube. 2µl of supernatant was used for dot blot. The supernatant of those oocytes which expressed target protein was kept in -20°C for use.

HbA buffer

Tris HCl (pH7.6)	20mM
$MgCl_2$	5mM
NaH_2PO4	5mM
EDTA	1mM
Saccharose	80mM

5.5 Methods about handling with DNA (buffer recipes included)

5.5.1 Isolation of genomic DNA from *C. reinhardtii*

The Isolation kit was made by Promega and the protocol was changed a little in this thesis. It is described as below:

The cells were grown to a density of 2×10^6 - 1×10^7 cells/ml and maximally 2×10^8 cells were used for the extraction. The cells were harvested and washed in 25ml 1×PBS (cold). The pellet was then resuspended in 600µl *Nuclei Lysis Solution* and transfered to a 2ml tube (At this step, cells could be kept in -80°C for a long time.).

The suspension was then incubated at 65°C for 15min and then cooled down to RT. 3µl *RNase-Solution* (RNase A, 4mg/ml) was added. The tube was kept at 37°C for 15 min. 250µlof Protein Precipitation solution was added and the suspension was vortexed. The tube was spun at full speed for 3 min in a microcentrifuge afterwards (If supernatant was not clear, it would be transferred to a new tube and spun again.).

Genomic DNA got precipitated by 700µl 100 isopropanol. The tube was centrifuged at full speed for 1 min. The pellet was washed with 600µl 70% ethanol. The DNA was dried in the air for 10-15 min and resuspended in 100-200µl of *Rehydration solution* at 65°C for 15 min.

5.5.2 Isolation plasmid-DNA from *E. coli*

A single colony picked from LB agar plate was inoculated into a test tube containing 5ml TB medium. The suspension was incubated in 37°C overnight with agitation (~250rpm). The cells were harvested the next day. Nucleospin Plasmid Kit (Macherey Nagel) was used to isolate plasmid DNA. Concentration and purity of DNA were determined by UV spectometer.

5.5.3 Agarose gel electrophoresis of DNA

TAE buffer was used routinely for agarose gel electrophoresis in this thesis. Ethidium Bromide (0.5ug/ml in agarose gel) was used to dye DNA. UV-lamp was used to observe DNA band. DNA samples were mixed with 6×loading buffer before loaded in the gel. Electrophoresis was carried out at constant voltage (5V/cm).

TAE buffer (pH8.0)

Tris-acetic acid	40mM
EDTA	1mM

6 × loading buffer

Glycerol	50%(v/v)
EDTA	7.5mM
Xylenxyanol	0.4%(w/v)
Bromophenol Blue	0.4%(w/v)

5.5.4 Purification of DNA from agarose gel

Necleospin Extract Kit (Macherey Nagel) was used to purify DNA fragment from agarose gel after electrophoresis.

5.5.5 Amplification of DNA fragment by Polymerase Chain Reaction(PCR)

A standard Polymerase Chain Reaction was carried out in 50µl volume with the ingredient below (Saiki et al., 1988).

10 × PCR buffer	5µl
10 × dNTP Mixture(2mM)	5µl
50pM Forward primer	1µl
50pM Reverse primer	1µl
Template-DNA	1µl(10ng)
Taq Polymerase	1U
DDW	up to 50µl

Because of the high GC content of Chlamy gene, 1M Betain was often added in the PCR reaction.

The standard temperature cycle is decribed as below:

Step 1.	95°C	5 min
Step 2.	94°C	30s
Step 3.	56°C-70°C	30s
Step 4.	72°C	1min/kb

25 cycles between Step 2 and Step 4

Step 5. 72°C 5min

Step 6. 4°C

PCR reaction could not only be used to amplify target DNA for cloning but to detect the *E. coli* transformants. Cell clumps picked from colonies on the plate could be directly added in the PCR reaction as templates. PCR could be also used to introduce mutation in target genes.

5.5.6 Digestion of DNA

Restriction enzymes were mainly purchased from NEB or MBI. Single restriction enzyme digestion or double restriction enzyme digestion were carried out after the direction of the manual.

5.5.7 Ligation of DNA

The ratio between vector DNA and target DNA were normally 1:3, which also varied in different cases. The T4 ligase was purchased from NEB and the ligation reaction was carried out according to the instruction.

5.5.8 *in vitro* synthesis of mRNA from DNA

RNA synthesis kit (mMESSAGE mMACHINE®)was purchased from Ambion and the process was carried out after manual instruction.

5.6 Method about handling with Protein (buffer recipes included)

5.6.1 Protein amount detection by BC Assay

The BC Assay (bioinchoninic acid) was a colorimeric assay, which involved the reduction of Cu^{2+} to Cu^{+} by peptidic bounds of proteins. The bioinchoninic acid chelated Cu^{+} with high specificity to form a water soluble purple color complex. Concentration known BSA (Bovine Serum Albumin) solution was used as standard. The kit was purchased from Interchim. The assay procedures were carried out after the manual instructions.

5.6.2 SDS-PAGE (SDS-Polyacrylamide Gel Electrophoresis)

SDS-PAGE was used to separate protein mixtures so that their relative abundances, purities and molecular weights could be determined. Mixed with detergent SDS (Sodium Dodecyl Sulphate) and 2-β-mercatoethanol, protein samples were heated to 95°C for 5 min for denaturation. Samples were driven by constant current of ~25mA for 1 hour.

Running Buffer

Tris	3.01g
Glycine	14.41g
SDS	1g

Dissolve in 1 l water

2×Sample buffer

Tris	65mM
SDS	3%(w/v)
Glycerol	30%(v/v)
β-mercatoethanol	5%(w/v)

Bromphenolblue	0.1(w/v)

5.6.3 Staining of SDS-PAG

5.6.3.1 Commassie Brilliant Blue staining

After electrophoresis, the SDS-PAG was taken out and soaked in staining solution for about 2-3 hours with constant agitation (RT). Then the colored gel was taken out and put into destaining solution with constant agitation. The destaining solution was changed several times till the protein band was clear.

Staining solution

Coomassie Brilliant Blue	0.2%
Methanol	45%(v/v)
Water	45%(v/v)
Acetic Acid	10%(v/v)

Destaining solution

Methanol	25%(v/v)
Water	65%(v/v)
Acetic Acid	10%(v/v)

5.6.3.2 Silver staining

After electrophoresis, the SDS-PAG was taken out and soaked in fixing solution (40% ethanol, 10% acetic acid) for 30 mines. Then the gel was put in incubation solution (30% ethanol, 0.2% sodiumthiosulfate, 500mM sodium acetate). Millipore water was used to wash the gel for three times (10 min each time). The gel was soaked in silver solution (0.1% silver nitrate, 0.02% formaldehyde) for 40 min. After shortly washed, the gel was put into developing solution (2.5% sodium carbonate (water free), 0.01% formaldehyde). 50mM EDTA (pH8.0) was used as stop solution when the bands were clear.

5.6.4 Western blot

To detect specific protein band, western blot was applied in this thesis. After SDS-PAGE, protein in the gel was transfered to a nitrocellulose membrane (Hybond-Amersham) by a semi-dry blotting system (Bio-Rad). The blotting process was carried out according to the manual instruction. After the transfer, the membrane was first blocked with blocking buffer and then incubated with blocking buffer containing the first antibody (4°C overnight or RT 1hour). The membrane was then washed for three times with blocking buffer, each time 10 min with constant agitation). Then blocking buffer with the second antibody was applied to the membrane

(4°C overnight or RT 1hour). The membrane was washed for three times with PBS-Tween solution for two times and once with PBS solution, each time 10 min with constant agitation. After that, the membrane was transferred to detection buffer. BCIP and NTB were added. The membrane was then washed in water to stop the reaction when the bands were clear.

Blotting Buffer

Tris	48mM
Glycine	39mM
SDS	0.1%

PBS(Phosphate Buffered Saline)

NaCl	150mM
Na2HPO4	16mM
NaH2PO4	1.8mM

PBS-Tween

PBS buffer with 0.05% Tween (v/v)

Blocking Buffer

7% Milkpowder in PBS-Tween solution

Detection Buffer

Tris · HCl	100mM
NaCl	100mM
MgCl2	50mM

5.6.5 In gel tryptic digestion

After Commassie Brilliant Blue staining or Silver staining, the band of target protein was cut out and sliced into 1mm× 1mm pieces. The pieces was collect in 1.5ml cup and sequentially washed with 50mM NH_4HCO_3, 50mM NH_4HCO_3 and 25%ACN, 25% ACN, 50% ACN. Every wash lasted for half an hour and the supernatant was discarded. The pieces were lyophilized for 1 hour.

The volume of the pieces was estimated and same volume of trypsinase solution (4µg in 100µl digestion buffer) was added. Then same amount of digestion buffer (50mM-100mM NH_4HCO_3) was added. The tube was kept in 37°C overnight.

The peptides of the digestion were extracted two times with 100 mM NH_4HCO_3 and one time with 40%ACN. Each extraction lasted for 1 hour and the extraction solutions volume were same as the volume of the pieces. All the extraction fraction was collected and lyophilized afterwards.

5.6.6 Purification with Ni-NTA column

Cells were harvested at 2,500 rpm for 5min and the pellet was resuspended in Buffer B (100ml cell culture in 1ml Buffer B). The suspension was centrifugated at 10,000g for 30min and the precipitation was discarded. The lysate supernatant was then loaded in the equilibrated column. The column was then washed two times with 3CV (column volume) Buffer C. Target protein was then washed off by adding 3 times 0.5 CV Buffer E.

Buffer B

Urea	8M
NaH2PO4	0.1M
Tris·Cl	0.01M, pH8.0

Buffer C

Urea	8M
NaH2PO4	0.1M
Tris·Cl	0.01M, pH6.3

Buffer E

Urea	8M
NaH2PO4	0.1M
Tris·Cl	0.01M, pH4.5

5.6.7 Purification with Strep-tactin column

Cells were harvested at 2,500 rpm for 5min and the pellet was resuspended in Buffer W (100ml cell culture in 1ml Buffer W). Cells were then broken by sonication. The suspension was centrifugated at 40,000g for 45 min. The supernatant was then collected and loaded in the equilibrated column. The column was then washed for 5 times with 1 CV (column volume) Buffer W. Target protein was then washed off by adding 6 times 0.5 CV Buffer E.

For regeneration of the column, the column was washed three times with 5CV Buffer R. When the column turned red, the column was overlaid with 2 ml Buffer R and kept in 4ºC for storage.

Buffer W (washing buffer):

Tris·Cl	100 mM
NaCl	150 mM
EDTA	1 mM pH 8

Buffer E (elution buffer):

Tris·Cl	100 mM
NaCl	150 mM
EDTA	1 mM
desthiobiotin	2.5 mM, pH 8

Buffer R (regeneration buffer):

Tris·Cl	100 mM
NaCl	150 mM
EDTA	1 mM
HABA (hydroxy-azophenyl-benzoic acid)	1 mM, pH 8.0

5.7 Materials

5.7.1 Strains

Strain	**Marker/Phenotype**	**Source**
E.coli DH5α	supE44,(Φ80lacZΔM15), hsdR17(r_K^-,m_K^+). recA1, endA1,gyrA96,thi-1,relA1, deoR Δ(lacZYA-argF)U169	Promega
C. reinhardtii cw15 arg- A	cw15, arg7, mt(-)	Rochaix &van Dillevijn, 1982
C. reinhardtii CC1930	arg2 mt(-)	Duke University, USA
C. reinhardtii CC645	pab2 mt(-)	Duke University, USA
C. reinhardtii CC124	wild type, mt(-)	Duke University, USA
C. reinhardtii CC125	wild type, mt(+)	Duke University, USA
C. reinhardtii CC621	wild type, mt(-)	Duke University, USA
C. reinhardtii CC620	wild type, mt(+)	Duke University, USA
C. reinhardtii CC477	bald 1	Duke University, USA
C. reinhardtii CC478	bald 2 mt(+)	Duke University, USA
C. reinhardtii CC479	bald 2 mt(-)	Duke University, USA
C. reinhardtii CC48	arg2, mt(+)	Duke University, USA
C. reinhardtii CC32	pab1, mt(+)	Duke University, USA
C. fusiformis		Nils Koeger
X. laevis		G. Nagel

5.7.2 Plasmids

Plasmid	**Marker**	**Source**
pBluescript II KS(-)	lacZ', amp^R	Stratagene
pSP109	zeo^R, amp^R	Stevens et al 1995
pSP124S	zeo^R, amp^R	Purton S
pArg7.8	zeo^R, arg 7	Debuchy et al 1989
pSI103	amp^R, aph VIII	Sizova I
pGen-D-Ble	zeo^R, amp^R	Fischer et al 2001
pGEM-RE	amp^R	Schiereis T.(Regensburg)
pPhot1	amp^R	Schiereis T.(Regensburg)
pBlue Cf FCPcass	amp^R	Nils Koeger
pUCBM20	amp^R	Peter Berthold
pXX212	amp^R	Markus Heitzer

5.7.3 Chemicals, Enzymes and Kits

5.7.3.1 Chemicals and Kits

Agar	Amresco
Agarose	BD-USA
Ampicillin	Sigma
L-Arginin	Degussa, Merk
Bacto-Trypton	Difco
Bacto-Yeast-extract	Difco
BC Assay	Uptima, Interchim
BCIP	Sigma
Coomassie Brillient Blue	Serva
dNTP mixture	MBI Fermentas
Ethidium bromide	Sigma
Glass beads	Braun Biotech
Gel Extraction Kit	Macherey Nagel
mMESSAGE mMACHINE	Ambion
NBT	Sigma
Ni-NTA Agarose	Qiagen
Ni-NTA spin column	Qiagen
Nitrocellulose	Schleicher&Schuell, Amersham
NP-40	Sigma
PEG 6000	Serva
Plasmid Extraction Kit	Macherey Nagel
Ponceau S	Sigma
TEMED	BioRad
Tween20	Sigma
Strep-Tactin agarose	IBA
Strep-Tactin AP conjugate	IBA
Zeocin	Invitrogen

All the other chemicals were purchased from Merck Company.

5.7.3.2 Enzymes and Proteins

Anti-BLE antibody	Cayla(French)
Anti-Phot1 antibody	T. Schiereis(Regensburg)
Anti-GFP antibody	M. Fuhrmann(Regensburg)
BSA	Sigma
CIP	Amersham
T4 DNA Ligase	MBI Fermentas
Vent Polymerase	NEB

All the restriction enzymes were purchased from MBI Fermentas.

5.7.4 Oligonucleotides

Oligonucleotides were purchased from Metabion.

5.7.4.1 Primers for making RNAi construct

Primers	**Sequences**
Puen1	AGCAAGCTTGCAGGGGTGCCAGCTCCA
Puen2	AGCGAATTCATGCGCACCGTGAGCTCC
Puen3	AGCGAATTCAGGTGAGTCGACGAGCAAGCCC
Puen4	AGCGGATCCCCTGCAAATGGAAACGGCGAC
Puen5	AGCGGATCCCCTCAACTACACCAAGGCCGG
Puen6	AGCTCTGAGCTTGGCCTTGGCCACCTCCT
Puh1	AGCGGTACCGCTGAGGCTTGACATGATTGGT
Puh2	AGCCTCGAGCATCCTGCAAATGGAAACGG
Puc1	AGCGAGCTCGCAGGGGTGCCAGCTCCA
Puc2(Puen6)	AGC TCTGAG CTTGGCCTTGGCCACCTCCT

The preparation of RNAi construct was shown as in Chapter 3.2.

5.7.4.2 Primers for oocyte expressions

Primers	**Sequences**
OP5XhoI	GCGCTCGAGATGGCAGGGGTGCCAGCT
LOV1-Ser5p	TGCTTGGTCACAACAGCCGCTTCCTCCA
LOV1-Ser3p	TGGAGGAAGCGGCTGTTGTGACCAAGCA
LOV2 SER 5	TGGGCCGCAACAGCCGCTTCCTGCA
LOV2 SER 3	TGCAGGAAGCGGCTGTTGCGGCCCA
Phot1-NotI-5	ATAGCGGCCGCGCCGCTGGGCGCCA
Phot1-NotI-3	ATAGCGGCCGCCGCCACCGCCGA
Phot1-3 BamHI	GCGGATCCTCAGTGGTAGTTGTCGAACGCCGCGCCGCCGGT
P5H8BamHISM	GCGGGATCCCACCACCACCACCACCACCACCACATGGCAGGGGTGCCAGCT
P3EcoRI	GCGGAATTCTCAGTGGTAGTTGTCGAACGCCGCGCCGCCGGT

C57S mutant was introduced by using two pairs of primers (OP5XhoI and LOV1-Ser3p, LOV1-Ser5p and

Phot1-NotI-3) and C250S mutant was introduced by using another two pairs of primers (LOV2 SER5 and Phot1-3 *Bam*HI, Phot1-NotI-5, LOV2 SER3). Phot1(C57S, C250S) was cloned into pXX20 between *Xho*I and *Bam*HI. pGEM-RE-Phot1(C57S, C250S) was made by cloning of PCR-generated Phot1(C57S, C250S) into pGEM-RE . PGEM-RE-Phot1 was constructed by cloning of PCR generated Phot1 into pGEM-RE.

5.7.4.3 Primers used for pLY2-phot1

Primers	**Sequences**
PsaD XbaI f	GCGTCTAGACACACACCTGCCCGTCTGCCTGACA
ble TEV BamHI r	GCGGGATCCGCCCTGGAAGTACAGGTTCTCGTCCTGCTCCTCGGCCACGAAGT
BamHI Phot1 f	GCGGGATCCGCAGGGGTGCCAGCTCCAGCCAGT
EcoRI Phot1 12 His r	GCGGAATTCTCAGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTAGTTGTCGAACGCCGCGCCGCCGGT
XhoI PsaD 3UTR f	GCGCTCGAGTTCTGGCAGCAGCTGGACCGCCTGT
KpnI PsaD 3UTR r	GCGGGTACCGCTGCATGTGCACAGTCACGCTGTCT
PsaD NdeI r	GCGCATATGGGCTTGTTGTGAGTAGCAGTGGGT
NdeI ble f	GCGCATATGGCCAGGATGGCCAAGCTGACCA

Plasmid pLY2 was generated by cloning of PCR-produced PsaD promoter-Ble fragment between *Xba*I and *Nde*I, and PsaD 3' UTR between *Xho*I and *Kpn*I into pBluescriptII KS-. Plasmid pLY2-Phot1 was constructed by cloning PCR-generated Phot1-12His fragment into pLY2 between BamHI and EcoRI sites.

5.7.4.4 Primers for diatom expressions

Primers	**Sequences**
DiBLEf	ATCAAAACAACCAAAATGGCCAAGCTGACCAGCGCCGT
StrepII Phot1r	TTACTTCTCGAACTGGGGGTGGCTCCAGTAGTTGTCGAACGCCGCGCCGCCGGT

Plasmid pDi-A was constructed by cloning of the PCR-generated *Ble-Phot1-Strep tag II* into pBlue Cf FCPcass. pLY2-Phot1 was used as templete. DiBLEf and StrepII Phot1r were used to amplify Ble-Phot gene. The vector pBlue Cf FCPcass was digested with *Eco*RV. The PCR product was cloned into the vector by blunt end cloning.

5.7.4.5 Primers for pLY2-A

Primers	Sequences
BamHIS12Phot1_5	GCGGGATCCCACCACCACCACCACCACCACCACCACCACCACGCAGGG GTGCCAGCTCCAGCCAGT
Phot1_3 XhoI	GCGCTCGAGTCAGGTCAGTCAGTAGTTGTCGAACGCCGCGCCGCCGGT

Plasmid pLY2-A was prepared from pLY2-phot1 by exchange of the *12His-Phot1* fragment for the *Phot1-12 His* fragment.

5.7.4.6 Primers for pLY2-B

Primers	Sequences
NdeIPhot1f	GCGCATATGGCAGGGGTGCCAGCTCCAGCCAGT
BamHI TEV BLE f	GCGGGATCCGAGAACCTGTACTTCCAGGGCGCCAAGCTGACCAGCGCCGT
BamHI His Phot1 rNS	GCGGGATCCGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTAGT TGTCGAACGCCGCGCCGCCGGT
XhoI Ble r	GCGCTCGAGTCAGTCCTGCTCCTCGGCCACGAAGT

Plasmid pLY2-B was constructed from pLY2-phot1 by exchange of the *Phot1-12His -TEV-Ble* fragment for the *Ble-TEV-Phot1-12His* fragment.

5.7.4.7 Primers for pLY2-C

Primers	Sequences
BamHI GFP f	GCGGGATCCGCCAAGGGCGAGGAGCTGTTCA
XhoI GFP r	GCGCTCGAGCTTGTACAGCTCGTCCATGCCGT
EcoRI Phot1f	GCGGAATTCGCAGGGGTGCCAGCTCCAGCCAGT

Plasmid pLY2-C was prepared from pLY2-phot1 by the exchange of the *GFP-Phot1* fragment for the *12His-Phot1* fragment.

5.7.4.8 Primers for pLY2-D, pLY2-E

Primers	Sequences
XhoI HA Phot1f	GCGCTCGAGATGTACCCCTACGACGTCCCCGACTACGCCGCAGGGGTGCCA GCTCCAGCCAGT
BamHI HA Phot1r	GCGGGATCCTCAGGCGTAGTCGGGGACGTCGTAGGGGTAGTAGTTGTCGA ACGCCGCGCCGCCGGT

Plasmid pLYM-D was constructed from pXX212 by the exchange of the *HA-Phot1-12His* fragment for the *Arg7-luc* fragment. Plasmid pLYM-E was constructed from pLY2-Phot1 by the exchange of the *12His-Phot1-HA* fragment for the *Arg7-luc* fragment.

5.7.4.9 Primers for Chapter 3.4.5.1

Primers	**Sequences**
NdeIStrepIIBLE	GCGCATATGTGGAGCCACCCCCAGTTCGAGAAGGCCAAGCTGACCAGCGCCGT
BamHIHISLOV2	GCGGGATCCCACCACCACCACCACCACCACCACCACCACCACCACTCGTTCCCGCGTGTGGCGCT
BamHIHISkinase	GCGGGATCCCACCACCACCACCACCACCACCACCACCACCACCACACCGCCAACCCCTGGGCGGCCA

Plasmid pLY2-SBP was prepared from pLY2-A by the exchange of the *Strep-Ble-TEV* fragment for the *Ble-TEV* fragment. Plasmid pLY2-SBL was prepared from pLY2-SBP by exchange the *12His-LOV2-kinase* fragment for the *12His-Phot1* fragment. Plasmid pLY2-SBK was prepared from pLY2-SBP by exchange the *12His-kinase* fragment for the *12His-Phot1* fragment.

Plasmid pLY2-BL was prepared from pLY2-A by the exchange of the 12His-Phot1 fragment for the *12His-LOV2-kinase* fragment. Plasmid pLY2-BK was prepared from pLY2-A by the exchange of the *12His-Phot1* fragment for the *12His-kinase* fragment.

5.7.4.10 Primers for pLY2-OV

Primers	**Sequences**
NdeIStrepIIspBLE	GCGCATATGTGGAGCCACCCCCAGTTCGAGAAGAUCAGCGGCGCCAACGGCGCCATGGCCAAGCTGACCAGCGCCGT
NdeIStrepIISP	GCGCATATGTGGAGCCACCCCCAGTTCGAGAAGATCAGCGGCGCGAACGGTGCA

Plasmid pLY2-OV was prepared from pLY2-A by the exchange of *Strep-spacer-Ble-TEV* fragment for the *Ble-TEV* fragment.

5.7.4.11 Sequencing primers

Primers	**Sequences**
SPH1	GCAGGGGTGCCAGCTCCAGCCA
SPH2	CGCACGATTGTGGACGACGTGA
SPH3	GGCCTTCTGGAACATGTTCACG

Primers	**Sequences**
SPH4	GCGATGAAGACGCTGGACAAGT
SPH5	TGCAGCTGGAGAACTACCTGCT
SPH1 reverse	GCGCAGGCGGTGGTCGTACTTG

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